

FUNCTIONAL DIVERSITY OF FUNGI ASSOCIATED WITH DURUM WHEAT ROOTS IN DIFFERENT CROPPING SYSTEMS

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ABSTRACT

Differences in pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) microbial compatibility and/ or their associated farming practices may influence root fungi of the following crop and affect the yield. The main objective of this research was to explain the difference in durum wheat (*Triticum turgidum* L.) yield the year after pea and chickpea crops through changes in the functional diversity of wheat root fungi. The effect of fungicides used on chickpea on the root fungi of a following durum wheat crop was studied using plate culture and pyrosequencing. Pyrosequencing detected more *Fusarium* spp. in the roots of durum wheat after fungicide-treated chickpea than in non-fungicide treated chickpea. Plate culture revealed that the functional groups of fungi responded differently to fungicide use in the field but the effect on total community was non-significant. Highly virulent pathogens were not affected, but antagonists were suppressed. More fungal antagonists were detected after the chickpea CDC Luna than CDC Vanguard. Fungal species responded differently to the use of fungicides *in vitro*, but the aggregate inhibition effect on antagonists and highly virulent pathogens was similar.

The effect of chickpea vs. pea previous crop and different chickpea termination times on root fungi of a following durum wheat crop was studied. The abundance of *Fusarium* spp. increased after cultivation of both cultivars of chickpea as compared to pea according to pyrosequencing and was negatively correlated with durum yield. Plate culture analysis revealed that fungal antagonists were more prevalent after pea than both cultivars of chickpea and chickpea CDC Vanguard increased the abundance of highly virulent pathogens. The abundance of highly virulent pathogens in durum wheat roots was negatively correlated to durum yield. Early termination of chickpea did not change the community of culturable fungi in the roots of a following durum crop.

It is noteworthy that *Fusarium redolens* was identified for the first time in Saskatchewan and its pathogenicity was confirmed on durum wheat, pea and chickpea. The classical method of root disease diagnostics in cereals is based on the examination of the subcrown internode. I evaluated the method by comparing the fungal communities associated with different subterranean organs of durum wheat. The fungal community of the subcrown internode was different from that of roots and crown, suggesting cautious use of this method.

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This thesis is dedicated to the true advocates for:

Freedom,

Human Rights,

Equality,

and Integrity.

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LIST OF ABBREVIATIONS

a.i.	Active Ingredient
ac	Acre
AC	Agriculture and Agrifood Canada
ADON	Acetyldeoxynivalenol
AM	Arbuscular Mycorrhiza
AMF	Arbuscular Mycorrhizal Fungi
ANOVA	Analysis of Variance
AR	Adventitious Roots
BLAST	Basic Local Alignment Search Tool
Bp	Base Pairs
C	Crown
C:N	Carbon : Nitrogen Ratio
CDC	Crop Development Centre
Df	Degree of Freedom
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DSE	Dark Septate Endophytes
EF	Elongation Factor
FABS	Food and Bioproduct Sciences
FAO	Food and Agriculture Organization
GLM	General Linear Model
ha	Hectare
ITS	Internal Transcribed Spacer
LSD	Least Significant Difference
Ls-Means	Least-squares Means
MANOVA	Multivariate Analysis of Variance
<i>N</i>	Total number of samples (plots) used in analysis
<i>n</i>	The number of values used to calculate the treatment mean
NCBI	National Center for Biotechnology Information
NMDS	Non-metric Multidimensional Scaling
NRC	National Research Council
Ns	Unspecified Nucleotides
NSERC	Natural Sciences and Engineering Research Council
OTU	Operational Taxonomic Group
PBI	Plant Biotechnology Institute
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
perMANOVA	Permutation Based MANOVA

ppm	Parts Per Million
Qo	Ubihydroquinone Oxidase Site
RNA	Ribonucleic Acid
s	Second
SAR	Systemic Acquired Resistance
SDHI	Succinate Dehydrogenase Inhibitor
SI	Subcrown internode
SMCD	Saskatchewan Microbial Collection and Database
SPARC	Semiarid Prairie Agricultural Research Centre
Sqs	Squares
SR	Seminal Roots
Syn	Synonym
USEPA	United States Environmental Protection Agency
w:v	Weight/Volume
WHO	World Health Organization

1. INTRODUCTION

A number of important grain legumes such as lentil, pea and chickpea that constitute a significant portion of human food diet are referred to as pulses (Duranti 2006). They belong to the family Fabaceae which is known to fix nitrogen through rhizobial symbiosis (Duranti 2006; Singh et al. 2007). Pulses are an important element of sustainable agriculture and an integral component of cropping systems since ancient times (Singh et al. 2007; Wani et al. 2003).

In recent decades, pulses have been incorporated into cereal-based cropping systems of the Canadian great plains (Knights 2004; Morrall 1997). They are usually used in rotation with cereals and much research has investigated their effects on plant health and crop productivity in these regions (e.g. Gan et al. 2009; Lupwayi and Kennedy 2007; Miller et al. 2003; Nayyar et al. 2009).

Chickpea is a high value crop, but ascochyta blight damage and poor wheat crops after chickpea as compared to other pulse crops such as pea are major risks associated with chickpea production in cereal-based cropping systems in Saskatchewan (Armstrong-Cho et al. 2008; Miller et al. 2002).

The rotation effects of different crops in agroecosystems varies because the biological characteristics of each crop and the management practices used to produce them are different (Kirkegaard et al. 2004; Yang et al. 2012b). For instance, a particular crop may leave more water and mineral nutrients for the subsequent crop (Miller et al. 2002). In fact, it was proposed that the soil after a pea crop contains more resources available for following crops than several other crops (Miller et al. 2002; Miller et al. 2003). However, difference in water and nitrogen availability did not fully explain the higher wheat yield measured after pea (Miller et al. 2002). Crop plants are known to modify the abundance of important fungi, which may affect the yield of the following crop (Fernandez 2007; Kirkegaard et al. 2004). Brassicas were shown

to increase the population of *Trichoderma* spp. while suppressing *Fusarium pseudograminearum* (Kirkegaard et al. 2004). Rotation effects on soil microbes may take place directly through the suppression or promotion of particular microorganisms by a previous crop or indirectly from the farming practices associated with that crop (Ahemad and Khan 2010; Prévost and Bromfield 2003; Yang et al. 2012b). Regardless of the mechanism, perhaps the most important goal of crop rotation is to suppress detrimental microbes and enhance beneficial plant associates (Baker and Cook 1974; Cook 2007; Janvier et al. 2007). Therefore, complementary explanations for the difference in wheat yield after pea and chickpea may be found in the rotation effects on plant health attributes. Accordingly, the main hypothesis of the research presented here is:

The microbial legacy of a chickpea crop promotes the health of a following crop of durum wheat to a lesser extent than the legacy of pea. This general hypothesis was further broken into smaller, testable hypotheses, including:

- The control of ascochyta blight on chickpea requires numerous foliar fungicide treatments. I hypothesize that fungicide applied on chickpea has non-target impacts on the functional diversity of fungi inhabiting the roots of durum wheat following in rotation, with impact on grain yield.
- Host identity is important in shaping the community of fungi associated with plants. I hypothesize that pea and different cultivars of chickpea differently influence the fungal community of the root endosphere and root health in a following crop with impact on yield.
- Chickpea matures much later than pea. This induces differences in soil physicochemical characteristics. I hypothesize that terminating chickpea at the same time as pea will eliminate the differences in the effect of chickpea and pea crops on the root mycota of a following crop of durum wheat.
- Fungi possess diverse physiological properties and tolerance to biocides. I hypothesize that the susceptibility of important fungi to fungicides differs, which explains the pattern of fungicide-driven changes in fungal communities observed in the field.

The objectives of this study were:

- Understanding the effects of fungicides on the community of endophytic and pathogenic fungi in the root system of durum wheat in relation to fungicides applied on a preceding chickpea crop.
- Determining the effect of chickpea genotypes on fungal communities associated with the root system of subsequent durum wheat.
- Determining the rotation effects of pea and chickpea on the community of fungi associated with the root system of subsequent crops of durum wheat.
- Evaluating the *in vitro* growth inhibition effect of different fungicides on most abundant and/ or functionally important fungi isolated from durum wheat roots *in vitro*.
- To reassess the accuracy of root rot disease diagnostics method in wheat.

2. LITERATURE REVIEW

2.1 Pulse Rotation in Cropping Systems of the Prairie

Crop rotation reduces the risk of plant disease outbreak due to accumulation of pathogens (Janvier et al. 2007) and improves soil microbial attributes (Biederbeck et al. 2005). Legumes are used as rotation crop in agroecosystems and their benefits to the cropping systems have been known to humans since ancient times (Halstead 1987). They provide agroecosystems with many advantages (Biederbeck et al. 2005; Kirkegaard et al. 2004; Miller et al. 2002). They are able to fix atmospheric nitrogen through the rhizobial symbiosis for their use and the subsequent crops also benefit from the recycling of this biologically-fixed nitrogen (Stevenson and Van Kessel 1996).

Chickpea (*Cicer arietinum*) is the third most important pulse in the world in terms of production (Pande et al. 2005). It was introduced to Saskatchewan during the 1990s (Knights 2004) where about 85% of Canada's chickpea production takes place (Gan et al. 2009). Chickpea has an indeterminate growth habit and end of season rainfall may sustain its growth into fall, increasing the risk of killing frost before maturity (Vandenberg 1998). Therefore, chickpea production in Saskatchewan is concentrated in the southwestern part of the province where drier conditions promote early maturity (Gan et al. 2009; Vandenberg 1998). Chickpea has high economic value and is tolerant to drought (Armstrong-Cho et al. 2008; Miller et al. 2001; Pande et al. 2005; Saskatchewan Ministry of Agriculture 2012), but inferior wheat productivity after chickpea compared to other pulses such as pea (Miller et al. 2002) is a major disadvantage.

Depending on their phytochemistry and phenology, different rotation crops affect soil biology, physics and chemistry in different ways (Cruz et al. 2012). For instance, chickpea matures later than pea and has deeper roots (Miller et al. 2001), which could mean that a chickpea crop leaves less water and minerals in the soil. Lower level of soil resources after

chickpea may reduce the yield of the crop which follows in rotation. Another major difference between pea and chickpea is the high susceptibility of chickpea to ascochyta blight caused by *Ascochyta rabiei* (Armstrong-Cho et al. 2008). Due to the lack of fully-resistant cultivars, chickpea production in western Canada is highly dependent on intensive fungicide treatment programs (Armstrong-Cho et al. 2008), including foliar spray and/ or seed treatment (Gan et al. 2006; Pande et al. 2005).

Despite the popularity of pulses in cropping systems in the Canadian prairie, little is known about their effects on subsequent crop (Miller et al. 2002). Miller et al. (2003) showed that pea rotation added more nitrogen to the soil and consumed less water than chickpea, but difference in post harvest soil water content had disappeared by the spring of the following year. Stevenson and Van Kessel (1996) suggested that a small portion of the benefits received by wheat from a previous crop of pea could be attributed to better soil nitrogen availability and that an important portion of the yield advantage brought about by pea could be attributed to improved wheat root health. Yang et al. (2012a) found that crop termination time influenced the composition of the bacterial communities associated with subsequent durum wheat (Yang et al. 2012a). They also showed changes in bacterial community structure due to foliar fungicide application on chickpea (Yang et al. 2012b). Termination time and fungicide use may also influence soil fungi.

Fungi are a very important component of agricultural systems everywhere (Olsson et al. 1999; Ruzicka et al. 2000; Schnürer et al. 1985) and can affect plant health and yield in positive and negative ways (Kirkegaard et al. 2004). Little is known about the effect of chickpea on the fungal communities associated with subsequent cereal crops in the Canadian prairie.

2.2 Fungi in Agroecosystems

Fungi constitute most of the microbial biomass of agricultural soils (Olsson et al. 1999; Ruzicka et al. 2000; Schnürer et al. 1985) and play a key role in ecosystem processes such as biogeochemical cycling (Anderson and Parkin 2007; Robinson et al. 2005). Some fungi grow on synthetic media (culturable fungi) but we are unable to grow some others using available media (non-culturable). Fungi interact with other biotic factors, i.e., the bacteria, animals, and plants,

affecting the functionality and productivity of the agroecosystem. Fungal interaction with plants is of great importance for agriculture. Based on the type of interaction they have with plants, the major groups of soil fungi can be grouped as: pathogens, mycorrhiza, root endophytes, and saprobes.

2.2.1 Pathogens

Fungi are the most important causes of plant diseases (Reeleder 2003) and they can result in huge economic losses by reducing crop yield (Armstrong-Cho et al. 2008; McMullen et al. 1997). The extent of damage caused by the pathogen to the host depends on genetics of host, and pathogen as well as the environmental conditions (Bos and Parlevliet 1995). Fungal pathogens may show different degrees of virulence on various hosts (Berbee et al. 1999; Bos and Parlevliet 1995; Esmaeili Taheri et al. 2011). For instance, a highly virulent pathogen could significantly damage or kill a host whereas a pathogen with lower virulence may only weaken the same host in a similar condition (Esmaeili Taheri et al. 2011). Similarly different plants may show different level of disease when attacked by a specific pathogen (Esmaeili Taheri et al. 2011).

Due to the economic importance of crop diseases caused by fungi, the vast majority of the research on the role and functionality of fungi in agricultural systems has been devoted to understanding the biology and ecology of disease-causing fungi and developing means to control them. Different tools have been used to minimize the impact of pathogens on crops such as resistant cultivars, fungicides, biocontrol and cultural practices such as rotation (Janvier et al. 2007; Pande et al. 2005).

The soil-borne diseases of plants are difficult to control mainly because the causative agents are hidden (Janvier et al. 2007). The control of soil-borne diseases relies primarily on cultural practices like crop rotation (Janvier et al. 2007). The management of the root and crown diseases of cereals is especially challenging due to the involvement of a wide range of pathogens (Sallans 1965), and the lack of resistance genes (Cook 2007). Furthermore, the definitions of the diseases and methodology of disease diagnostics are inconsistent which may lead to confusion and add to the difficulties of cereal root and crown disease management. For instance, many

researchers reported several species such as *Cochliobolus sativus* and *Fusarium* spp. as the potential causative agents of common root rot (e.g. Burrage and Tinline 1960; Chen et al. 1996; Hill et al. 1983; Sallans 1965; Tobias et al. 2009); however, others limited the use of this name where the dominant agent is *C. sativus* and applied other names such as “Crown rot” or “Fusarium root rot” when *Fusarium* spp. are dominant (e.g. Fedel Moen and Harris 1987; Smiley et al. 2005; Wiese et al. 2000). Furthermore, the techniques used for the quantification of the diseases and the isolation of causal agents in the cereal root and crown disease complex vary among researchers. Different subterranean organs of wheat (i.e. seminal roots, adventitious roots, subcrown internode and crown) are used for the isolation of fungi and the quantification of disease severity (e.g. Chen et al. 1996; Diehl et al. 1982; Fernandez et al. 2007; Gonzalez and Trevathan 2000). The anatomy (Bailey et al. 2003; Li et al. 2008) and physiology of the different subterranean organs of cereals are different and these organs are placed at different soil depths, which may affect the associated fungal communities. Inconsistencies in the definition and methodologies used to quantify the diseases and isolate causative agents are a source of confusion that makes understanding and managing soil-borne root and crown diseases of cereals even more challenging.

2.2.2 Arbuscular Mycorrhizal Fungi

The roots of the vast majority of terrestrial plants are colonized by mycorrhizal fungi, which benefit their host in many ways (Jeffries and Rhodes 1987; Smith and Read 1997). A few types of mycorrhizal symbioses have been defined. The arbuscular mycorrhizal (AM) symbiosis involves the fungi of the Glomeromycota which are obligate symbionts (Schüßler et al. 2001). The AM symbiosis is of great interest in agriculture because of their abundance in agricultural soils and their mutualistic association with important crop plants (Olsson et al. 1999; Smith and Read 1997). The AM symbiosis helps plants in many different ways. It improves the mineral nutrition (e.g. P, Zn, Ni, Cu) of plants and their tolerance to root pathogens and abiotic stresses (Graham and Menge 1982; Jeffries and Rhodes 1987; Smith and Read 1997). Graham and Menge (1982) have shown improved phosphorus absorption and reduced take-all disease in wheat as a result of inoculation with *Glomus fasciculatus* and Allen and Boosalis (1983) have shown improved drought tolerance in wheat associated with this fungus. The AM fungi can

functionally complement each other (Jansa et al. 2008) explaining why AM fungal diversity is important in determining the outcome of the symbiosis (van der Heijden et al. 1998).

Since the effects of the mycorrhizal symbiosis on the host plant are usually positive, the goal of scientists and practitioners is to maximize their benefits in crop production through appropriate management practices.

2.2.3 Endophytes

Endophytic fungi belong to various taxonomic groups of the kingdom Fungi (Rodriguez et al. 2009). They are characterized by the fact that they colonize plant tissues asymptotically (Wilson 1995). Some fungal endophytes can benefit plants. They may improve plant mineral nutrition by enhancing phosphorus (Newsham 1999) and nitrogen availability (Mandyam and Jumpponen 2005; Newsham 1999). Disease protection is another service provided by some endophytic fungi to their host as has been shown in many studies (Lahlali and Hijri 2010; Ownley et al. 2008). The inoculation of plants with fungal endophytes may improve host tolerance to abiotic stresses (Khan et al. 2011; Li et al. 2012; Shukla et al. 2012). A group of dark pigmented endophytic fungi are called dark septate endophytes (DSE) which are specialized in colonizing roots (Rodriguez et al. 2009). They are suggested to be prevalent in different habitats (Mandyam and Jumpponen 2005). Similar to the AM fungi, the goal in sustainable agriculture should be to maximize the benefits of these fungal endophytes.

2.2.4 Saprotrophs

Saprotrophic fungi are fundamental elements in nutrient cycling (Robinson et al. 2005). This group of fungi is involved in organic matter decomposition in soil, freeing up nutrients from biomass in a process that is particularly important for plants (Miller 1995). While all fungi are important in nutrient cycling, Basidiomycota possess the enzymatic capabilities to degrade complex compounds like lignin, and therefore are a key element in the process of soil organic matter decomposition (Frankland et al. 1982; Robinson et al. 2005; Thormann 2006). Saprotrophic fungi are sensitive to environmental changes and pollutants that may affect the decomposition process with impact on ecosystem functionality (Robinson et al. 2005). However,

very little is known of the effect of such disturbances on saprophytic fungal communities (Stromberger 2005).

2.3 Suppressive Soils

Suppressive soils either prevent the establishment of pathogens, or prevent established pathogens from causing disease (Baker and Cook 1974). Soil suppressiveness may be due to the presence of specific microbial antagonists (Hoitink and Fahy 1986). Several fungal taxa are known to antagonize soil pathogens such as *Trichoderma* and *Gliocladium* (Hoitink and Fahy 1986; Papavizas 1985). Due to the difficulty of controlling soil-borne root diseases using classical disease control methods, tackling these disease through increased soil suppressiveness is an attractive option (Janvier et al. 2007). Cultural practices such as crop rotation, organic amendments and tillage can be tools for improving soil suppressiveness (Baker and Cook 1974; Janvier et al. 2007).

2.4 Niche Specialization in Soil Fungi

Fungi are heterotrophs and thus are dependent on the availability of organic carbon sources and often specialize into specific nutritional niches (Lowe and Howlett 2012). The AM fungi are obligate biotrophs but the nutritional niches of other fungi may vary from obligatory biotrophs to completely free-living saprotrophs (Alexopoulos and Mims 1979; Lowe and Howlett 2012; Schüßler et al. 2001). Nutritional niches shape many aspects of fungal life, including winter survival (Garrett 1951). For instance, the fungi that are highly specialized into parasitizing root systems have lost much of their ability to live freely on soil organic matter (Garrett 1951). They possess specialized resting spores to survive periods of unfavorable conditions (Menzies 1963) or became stubble-borne, which means they can survive in the previous year plant residues before attacking the next crop (Bockus and Shroyer 1998).

2.5 Methods of Studying Microbial Communities

Due to the complexity in methodology, incomplete taxonomic knowledge and the unculturable nature of the majority of microorganisms, understanding microbial ecology is challenging (Kirk et al. 2004; Torsvik et al. 1998). New techniques have overcome many

historical limitations associated with classic culture-based techniques, but they also have limitations (Drenovsky et al. 2008). To obtain a comprehensive picture of microbial communities, it is recommended to use multiple complementary techniques simultaneously (Kirk et al. 2004). The outcome of microbial community studies can be influenced by the method used (Robinson et al. 2005); therefore, a sound understanding of the strengths and weaknesses of each technique is critical. Methods for studying fungal communities are grouped into two main categories: culture-dependent and culture-independent methods (Hill et al. 2000).

2.5.1 Culture-Dependent Methods

In culture-dependent methods, microbes are grown on a synthetic medium and their abundance is quantified based on colony number (Kirk et al. 2004). An important advantage of culture-dependent techniques is that they provide living fungus for further experimentation and application. Newer culture-dependent methods such as Sole Carbon Source Utilization Pattern analysis are also available to obtain a picture of the physiological profile of the microbial community (Garland and Mills 1991; Kirk et al. 2004). However, the main limitation of culture-dependent techniques is that the vast majority of microbes do not grow on synthetic media (Tedersoo et al. 2010; Torsvik et al. 1998).

2.5.2 Culture-Independent Methods

To overcome the limitations of culture-dependent methods, several molecular and biochemical methods have been developed and are increasingly used (Hill et al. 2000; Kirk et al. 2004). These are based on variation in specific molecules (i.e. DNA, RNA or phospholipid fatty acid) to produce a ‘fingerprint’ of the microbial community. Fingerprints of different communities can be compared (Drenovsky et al. 2008). A wide spectrum of culture-independent techniques are available (Hill et al. 2000; Kirk et al. 2004). Some of the most important ones are described below.

2.5.2.1 Phospholipid Fatty Acid Profiling

In this method, the phospholipid fatty acids (PLFA) of biological membranes are used to produce a fingerprint of microbial communities (Hill et al. 2000). Certain fatty acids are used as indicators of broad taxonomic groups (Hill et al. 2000). Because membranes usually degrade very fast after cell death, this technique was proposed as a reliable indicator of active microorganisms (Hill et al. 2000). PLFA profiling is a reproducible method used to compare the structure of total active microbial communities (Haack et al. 1994). However, the PLFA method does not provide precise taxonomic information (Hill et al. 2000) and also it is suggested that the outcome of this method may be highly influenced by the environmental conditions in which the target microbes are grown (Haack et al. 1994).

2.5.2.2 Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is a reproducible, fast and inexpensive PCR-based method for scanning the changes in fungal community structure (Gelsomino et al. 1999; Kirk et al. 2004). Many people have used DGGE to study the community structure of general soil fungi (e.g. Anderson et al. 2003a; Bastias et al. 2006; van Elsas et al. 2000) or AM fungi (Liang et al. 2008; Öpik et al. 2003). However, this method is inadequate to detect rare members of microbial communities (Gelsomino et al. 1999; Muyzer and Smalla 1998) and lacks sensitivity and discrimination power (Gelsomino et al. 1999), which could be a source of error. Also, like all other PCR-based methods, DGGE could be affected by PCR inhibitors such as humic acids (Kirk et al. 2004) and cell lysis efficiency (Prosser 2002).

2.5.2.3 Pyrosequencing

In 1998, Ronaghi et al. (1998) introduced pyrosequencing, which is a revolutionary technique for sequencing DNA. Metagenomic pools of amplicons can be sequenced simultaneously, eliminating the need for a pre-sequencing amplicon separation step (Ronaghi et al. 1998). Pyrosequencing is very effective in detecting fungal diversity (Buée et al. 2009). It is a high-throughput, semi-quantitative method (Sinclair et al. 2003), which is a critical asset for microbial ecology studies since it can be used to assess treatment effects (Sugiyama et al. 2010). Limitations associated with this method are short sequence read lengths and lack of precise

datasets for sequence comparison (Bridge et al. 2003; Haas et al. 2011). Pyrosequencing of amplicons is also subject to PCR-related limitations that include the bias created by preferential amplification of some taxa, PCR inhibitors and the efficiency of cell lysis during DNA extraction (Kirk et al. 2004; Prosser 2002).

2.6 Primer Selection in Molecular Biology Techniques

Primer selection is critical in molecular studies of microbial communities (Jumpponen 2007; Schmalenberger et al. 2001). Primer selection is based on the target microbial communities (Redecker et al. 2003). The small subunit of the ribosomal gene in Ascomycota and Basidiomycota is known to carry insufficient variation to differentiate fungi at the species level (Anderson and Cairney 2004; Tedersoo et al. 2010). The internal transcribed spacer (ITS), on the other hand, provides appropriate variation for species-level identification, and databases are rich in reference sequences using this region (Anderson and Cairney 2004; Anderson et al. 2003b; Anderson and Parkin 2007; Nilsson et al. 2009). Therefore, the ITS region is widely-used in fungal ecology studies (e.g. Anderson and Parkin 2007; Buée et al. 2009; Ovaskainen et al. 2010; Xu et al. 2012). Due to sequence length limitations imposed by available pyrosequencing techniques and DGGE, it is not possible to use the whole ITS region in ecological studies and researchers have amplified either the ITS1 or ITS2 region to cope with the limits of both pyrosequencing and DGGE (Anderson et al. 2003a; Buée et al. 2009; Mello et al. 2011). Mello et al. (2011) have compared the efficiency of ITS1 and ITS2 and found that both regions are adequate for the analysis of communities, although ITS2 yielded more non-fungal hits. While longer DNA sequences provide better resolution necessary for identification, some researchers have suggested that a DNA sequence of about 250 bp would be sufficient to identify fungi at the species or genus level (Buée et al. 2009).

2.7 Crop Management Practices and their Effect on Soil Fungi

Crop management practices affect the fungal communities in soils and plants, which in turn may affect yield (Alguacil et al. 2008; Fernandez 2007; Kirkegaard et al. 2004). The effects of farming practices on the fungal communities of agricultural soils is not sufficiently understood

(Stromberger 2005). Knowledge of the effects of farming practices on the communities of pathogenic and beneficial fungi related to this thesis are summarized in the following sections.

2.7.1 Rotation

Different crops differ in phenology, root exudation (Gransee and Wittenmayer 2000), consumption of soil nutrients and water, rooting pattern, phytochemistry (Cruz et al. 2012), and defense compound profiles (Hadwiger 2008; Stevenson et al. 1997; Ye et al. 2002). Consequently, the microbial compatibility of each crop is unique. For example, the cognate rhizobacteria for chickpea is *Mesorhizobium*; whereas, pea forms symbioses with the genus *Rhizobium* (Ahemad and Khan 2010; Prévost and Bromfield 2003). Consequently, each crop may leave a unique microbial heritage to the following crop(s). Kirkegaard et al. (2004) have shown that the chickpea legacy can include high populations of *F. pseudograminearum*, which may decrease the productivity of a subsequent wheat crop. They suggested that the high abundance of the pathogen could be related to the decreased prevalence of antagonistic *Trichoderma* spp. Based on the knowledge of the pathogenic fungi associated with different crops in Saskatchewan, Fernandez (2007) predicted increases in populations of Fusaria with the incorporation of broadleaf crops in cropping systems. The inclusion of pulses in rotation was shown to increase the abundance of *F. avenaceum* in the subcrown internodes of wheat; whereas, cereals or summer fallows increased the population of *C. sativus* (Fernandez et al. 2007). Stevenson and Van Kessel (1996) reported a decreased incidence of common root rot in wheat when pea was incorporated in the system, compared with continuous wheat. However, it is worth noting that due to the limitations in studies of cereal root and crown diseases mentioned in section 2.2.1, the actual effects of management practices, including rotation on cereal root health, is poorly understood.

2.7.2 Fungicide Use

Biocides are applied widely to control specific pests and diseases in crops, and they may affect non-target organisms (e.g Assaf et al. 2009; Chakravarty and Chatarpaul 1990; Schreiner and Bethlenfalvay 1996; Yang et al. 2012b). It is known that the application of fungicides changes the structure and species composition of the soil microbiota (Schreiner and

Bethlenfalvay 1996). Assaf et al. (2009) have shown that fungicide application may reduce the production of arbuscular mycorrhizal fungi (AMF) spores in chickpea roots. Wilson et al. (2008) showed that the fungicide propiconazole affected the fungal endophytes of *Hordeum brevisubulatum*. Hill and Brown (2000) also showed that systemic fungicide use can reduce the endophytic fungal colonization of grasses. The effect of fungicides may take place through direct growth suppression or indirectly through affecting other biological components of the system such as plants (Chiocchio et al. 2000; Cruz et al. 2012; Yang et al. 2011; Zambonelli and Iotti 2001).

2.8 Control of Ascochyta Blight in Saskatchewan

Ascochyta blight (*Ascochyta rabiei*) is probably the most important disease of chickpea worldwide (Armstrong-Cho et al. 2008; Gaur and Singh 1996; Pande et al. 2005). Some genotypes of chickpea possess partial resistance to the disease, but fungicide application on aerial parts or as seed dressing is often necessary to prevent outbreaks of ascochyta blight (Armstrong-Cho et al. 2008; Gan et al. 2006; Pande et al. 2005). A few fungicides such as chlorothalonil, azoxystrobin, pyraclostrobin and boscalid are registered for the control of ascochyta blight in Canada (Armstrong-Cho et al. 2008). These fungicides target specific metabolic elements (Avenot and Michailides 2010; Bartlett et al. 2002; Tillman et al. 1973) and their effects on different members of fungal community differ. In my thesis, the effects of boscalid, chlorothalonil and pyraclostrobin on plant-associated fungi were examined. Therefore, the modes of action of these fungicides are discussed in the following sections.

2.8.1 Boscalid (Lance)

Boscalid is a broad-spectrum systemic fungicide belonging to the succinate dehydrogenase inhibitor (SDHI) group of fungicides (Avenot and Michailides 2010; FAO and WHO 2009). It targets the mitochondrial complex II and thus inhibits fungal respiration (Avenot and Michailides 2010). Boscalid is considered a fairly persistent compound in nature (Lagunas-Allué et al. 2010; Reilly et al. 2012), which raises concern about its prolonged effects on the environment.

2.8.2 Pyraclostrobin (Headline)

Pyraclostrobin belongs to the group of strobilurin fungicides that were first marketed in 1996 and rapidly became an important group of fungicides (Bartlett et al. 2002). Strobilurin fungicides stop respiration by binding to the Qo site of cytochrome b (Bartlett et al. 2002). This mode of action is completely new and distinct from previously-known respiration inhibitors; therefore, the risk of cross-resistance to old fungicides is minimal for strobilurins (Bartlett et al. 2002). High-energy demanding processes like spore germination and zoospore motility are very sensitive to strobilurin fungicides (Ammermann et al. 2000; Bartlett et al. 2002; Karadimos et al. 2005). Strobilurin fungicides are considered safe for the environment due to their relatively-fast degradation (Balba 2007; Bartlett et al. 2002). Strobilurin fungicides possess a wide range of mobility in plants, but pyraclostrobin is local systemic (Bartlett et al. 2002; BASF).

2.8.3 Chlorothalonil (Bravo)

Chlorothalonil (Bravo) is a multi-site contact fungicide (Syngenta Crop Protection Pty Limited ; Wyss et al. 2004), widely-used for the control of foliar diseases (Sigler and Turco 2002). Chlorothalonil inhibits respiratory and glycolytic enzymes (Tillman et al. 1973). The half-lives of chlorothalonil in peanut farm soil has been shown to be between <1 and 3.5 days (Potter et al. 2001).

2.9 Fungal Biodiversity Studies in Agricultural Systems in South Western Saskatchewan

Fernandez and Jefferson have studied the population of fungi invading crown and subcrown internode of wheat in Saskatchewan and found that in brown soil *C. sativus* was more abundant whereas in black and dark brown soils more *Fusarium* spp. were observed (2004). Fraser (2008) studied the effect of pulse crop rotation on AM fungal root colonization in Swift Current, Saskatchewan. She found different levels of AM fungal colonization in durum wheat roots following different crop plants, but did not detect any changes in microbial community structure using PLFA markers. Mavragani (2008) studied the changes in abundance of fungal pathogens in durum wheat in relation to cropping history, also in Swift Current, and reported a lower frequency of *Bipolaris sorokiniana*, a pathogen causing common root rot, after pea than other rotation crops. Dai et al. (2012) have also studied the abundance and diversity of AM fungi

in different soil types in Saskatchewan using the small subunit of the RNA ribosomal genes and found the highest AM fungal diversity in Black Chernozem soils.

3. FUNGICIDE APPLICATION ON CHICKPEA IMPACTS THE FUNGAL COMMUNITIES LIVING IN THE ROOTS OF DURUM WHEAT FOLLOWING CHICKPEA IN THE CROPPING SYSTEM

3.1 Abstract

In wheat-based cropping systems of Saskatchewan, wheat yield after a chickpea crop is lower than after pea. Intense foliar fungicide application is necessary for chickpea production due to the susceptibility of this crop to ascochyta blight. Soil fungi are involved in several key functions of the agro-ecosystems such as nutrient turnover, mutualistic and pathogenic relationship with plants. Fungicide application may have non-target effects on soil fungi that consequently could alter system productivity and yield. The fungicide abundantly applied on chickpea to control ascochyta blight may impact the fungal functional diversity and feedback on the productivity of durum wheat following in rotation. The effects of foliar fungicide application on chickpea CDC Vanguard and CDC Luna on the fungal communities associated with the root system of a following durum wheat crop were studied in a 2-year field experiment using plate culture and pyrosequencing. Culturable fungal endophytes (colonizing asymptomatic roots) and pathogens (invading symptomatic roots) were isolated separately to have a precise understanding of the effects on fungal endophytes and pathogens. The effect of fungicide on the structure of the fungal community inhabiting the wheat root endosphere depended on year and root type. Pyrosequencing of durum wheat roots at heading revealed that *Fusarium* abundance was positively associated with fungicide application on previous chickpea. Fungicide applied on chickpea reduced the relative abundance of the culturable fungal antagonists colonizing durum roots in the following year while the overall population of culturable pathogenic species remained unchanged. Chickpea CDC Luna increased the abundance of culturable fungal antagonists compared to CDC Vanguard. Fungicide use on chickpea appears to preferentially favor the establishment of fungal pathogens over antagonists in the root endosphere of a following wheat crop, which may impact grain yield.

3.2 Introduction

Pulses provide many advantages in cereal-based cropping systems (Kirkegaard et al. 2004; Miller et al. 2002); however, the benefits of different pulses to the following crop differs (Miller et al. 2003). In Canadian prairie regions, for example, it has been shown that wheat yield is usually lower after chickpea (*Cicer arietinum* L.) than pea (*Pisum sativum* L.) (Miller et al. 2002). Chickpea is highly susceptible to ascochyta blight caused by *Ascochyta rabiei* and chickpea production is heavily dependent on foliar fungicide use (Armstrong-Cho et al. 2008; Gan et al. 2006). Fungi are involved in several key functions that affect plant productivity directly (e.g. pathogenic or mutualistic interactions) or indirectly (e.g. nutrient turnover) (Anderson and Parkin 2007; Jeffries and Rhodes 1987; Reeleder 2003; Robinson et al. 2005). The fungicides used to control diseases in agriculture can have non-target effects impacting soil fungi including functionally-important groups such as arbuscular mycorrhizal fungi (AMF) (Assaf et al. 2009; Schreiner and Bethlenfalvay 1996) and consequently affect plant productivity. However, our understanding of the non-target effects of fungicides on microbial diversity (Sigler et al. 2000; Sigler and Turco 2002) and therefore on the functionality of agroecosystem is limited.

The goal of this study was to determine the effects of fungicide use on chickpea on the fungal communities associated with the roots of a following durum wheat crop. Culture-dependent (plate culture) and culture-independent techniques (pyrosequencing) were used. Fungal communities occupying symptomatic versus asymptomatic roots were analyzed separately in plate culture to examine the fungicide effects on root endophytes and pathogens precisely because the function and interaction of endophytes and pathogens with the plant are different (Wilson 1995). In pyrosequencing the adventitious versus seminal roots were examined independently to account for differences in phenology and their position in the soil.

I hypothesized that fungicide application on chickpea plants can influence the fungal communities inhibiting the endosphere of a subsequent durum crop and increase the population of detrimental fungi and/or decrease that of beneficial species with possible impact on subsequent yield.

Since chickpea is often grown in cereal-based cropping systems in semiarid regions of the Canadian prairie (Cruz et al. 2012), it is important to understand the influences of this crop on the microbial components of the soil.

3.3 Materials and Methods

3.3.1 Experimental Design and Management

The experiment was established at the Semiarid Prairie Agricultural Research Centre in Swift Current, Saskatchewan (latitude 50° 18' N; Longitude 107° 41' W). Chickpea grown in 2008 was followed by durum wheat in 2009, and the experiment was repeated in 2009-2010. The experiment had a 2 x 2 factorial split plot design with four replicates. There were two levels of chickpea cultivars randomized in main plots: 1- CDC Vanguard and 2- CDC Luna, and two levels of fungicide randomized in subplots, 1- non-treated control and 2- five fungicide applications. Fungicide treatment started 4-5 weeks after seeding and repeated every 10-14 days thereafter. Two products were used; Bravo (Syngenta Crop Protection Canada Inc., Guelph, ON, active ingredient (a.i.) chlorothalonil, 1 kg ha⁻¹) was applied three times and Headline Duo (BASF Canada Inc., Mississauga, ON, a.i. pyraclostrobin and boscalid 100 g ha⁻¹ and 240 g ha⁻¹, respectively) was applied twice. Fungicides were applied in following order: Headline Duo, Bravo, Headline Duo, Bravo and Bravo.

January to the end of August average precipitation per month in 2008 was 46.38 mm, in 2009 was 28.38 mm, and in 2010 was 60.88 mm. Durum wheat AC Avonlea was seeded on May 5th in 2009 and on May 13th in 2010 at a seeding rate of 113 kg ha⁻¹. Row spacing was 25.4 cm and plot size 2 m x 8 m. Seeds were treated with fungicidal product VitaFlo 280 at 330 mL 100 kg⁻¹. Roundup Weathermax (Monsanto Canada Inc, Winnipeg, Manitoba, Canada) was applied not later than a day after seeding at a rate of 816.8 mL ha⁻¹. Twenty two kg ha⁻¹ actual phosphorus and 56 kg ha⁻¹ actual nitrogen were added to the plots. Crops were harvested on August 28th in 2009 and on September 27th in 2010.

At the early heading stage, the communities of seminal and adventitious roots of durum wheat after one chickpea cultivar (CDC Vanguard) were determined using pyrosequencing.

Seminal and adventitious roots were analysed separately to account for differences in time of emergence and position in the soil of the two root types. Plate culture was used to quantify the communities of culturable endophytic and pathogenic fungi associated with asymptomatic and symptomatic durum roots at maturity after two cultivars of chickpea (CDC Luna and CDC Vanguard) (Wilson 1995). Using different methods in monitoring microbial communities is recommended to provide a more comprehensive picture of the community (Kirk et al. 2004).

3.3.2 Root Sampling and Processing

Thirty to forty durum wheat plants were dug up to a depth of about 20 cm in each plot at the early heading stage (early July) and at maturity (August-September). Sampling at maturity was done to yield maximal root fungal biodiversity; whereas, at the early heading stage, the plant is still physiologically active and the associated fungi in that stage could be more involved in plant productivity. Shoots were cut 4 cm above ground level; whereas, roots were washed in tap water and stored at 4°C. The roots of wheat at the early heading stage were dried at 60°C for 72 h. Dry seminal and adventitious root materials were ground separately in 1.5 mL microcentrifuge tubes with three tungsten beads for 2-4 min in the Retsch® MM301 (Haan, Germany) bead miller, using stroke frequency of 25 s⁻¹. DNA was extracted from 28 mg of ground samples using Qiagen DNeasy plant DNA extraction kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol.

The fungal colonization of mature plant roots were examined through plate culture. Root plating followed the procedure described by Fernandez and Jefferson (2004) with some minor modifications. Segments of symptomatic and asymptomatic roots of various diameter and 8 mm in length were plated on a solid medium made of 5 g L⁻¹ Potato Dextrose Agar (PDA) (Becton, Dickinson And Company, Sparks, MD, USA) and 12 g L⁻¹ Bacto-Agar (Difco Laboratories Inc, Detroit, MI, USA). 10 mL L⁻¹ 0.6% chlortetracycline (Sigma-Aldrich, St. Louis, MO, USA), and 1.5 mL L⁻¹ 0.3% streptomycin sulfate were added to the medium to prevent bacterial growth (Sigma-Aldrich, St. Louis, MO, USA). Root surface sterilization was done by sequential soaking in 96% ethanol (30 s), 20% commercial Javex (6% w:v sodium hypochlorite; 90 s), and rinsing in sterile distilled water four times (Burgess et al. 1988; Fernandez and Jefferson 2004; Vujanovic et al. 2002). The total number of plates and root segments was equal in 2009 and 2010

but in 2009, seminal and adventitious roots were not separated; whereas, in 2010 they were plated independently. Plates were stored in the dark at room temperature and emerging fungi were subcultured on PDA (Becton, Dickinson And Company, Sparks, MD, USA) amended with 1.5 mL L^{-1} 0.3% streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (Vujanovic et al. 2002).

3.3.3 PCR and Preparation of Amplicons for Pyrosequencing

A fragment of the ITS region about 400 bp in length was amplified using fusion primers ITS1-F / ITS2 (Table 3.1) (White et al. 1990). Thirty-two unique multiplex identifiers of 10 bp were used to label the sequences of each sample (Dai et al. 2012; Roche 2009). The DNA extracts for each plot were subjected to two PCR reactions of 21.6 μL Platinum PCR Supermix High Fidelity (Invitrogen, Carlsbad, CA, USA), 3 μL Bovine serum albumin (Bio-Rad), 7 μL of DNA and 1.2 μL of each primer (20 pmol) (Anderson et al. 2003a) in a 96 well PCR thermocycler machine (Eppendorf AG, Hamburg Germany). PCR conditions were: 94 °C for 3 min, 34 cycles at 94 °C for 1 min, 54°C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min, as modified from Mavragani (2008). The tagged amplicons obtained were pooled to produce one sample per plot (Dai et al. 2012; Yang et al. 2012b) prior to purification using QIAquick PCR purification kits (Qiagen Inc., Valencia, CA, USA), according to the protocol of the manufacturer. The DNA concentration was measured in a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) using Quant-iT™ Assay Kits (Invitrogen, Carlsbad, CA, USA) (Schellenberg et al. 2011) and adjusted to $20 \text{ ng } \mu\text{L}^{-1}$. Equimolar amounts of 6 μL aliquots of the 32 purified tagged amplicon mixes were pooled and sent to NRC for pyrosequencing with Roche 454 flx titanium technology in one eighth of reaction (Dai et al. 2012; Yang et al. 2012b).

3.3.4 Sequence Management, Clustering and Taxonomical Annotation

Primer sequences were trimmed from the sequences received from NRC using BioEdit (Hall 1999). The online pipeline CLOTU (<http://www.biportal.uio.no>) (Kumar et al. 2011) was used for clustering and BLAST of the sequences. Sequences shorter than 170 bp or containing unspecified nucleotides were excluded from the analysis (Hui et al. 2011) and homopolymers longer than 8 were collapsed. Sequences were clustered into Operational Taxonomic Unit (OTU)

at 97% sequence identity and 85% sequence coverage using BLASTCLUST (Altschul et al. 1997; Hui et al. 2011; Jumpponen and Jones 2009). The longest sequence from each cluster was BLASTed against NCBI using CLOTU (Kumar et al. 2011). Singletons, OTU represented with only one read (Tedersoo et al. 2010), were excluded from the analysis (Dai et al. 2012).

Parameters that are not specified here were kept as CLOTU defaults

(<http://www.bioportal.uio.no>).

3.3.5 DNA Extraction, PCR and Fungal Identification

The fungal cultures obtained from the root samples were grouped in OTUs based on morphology to reduce the number of PCR reactions. At least one representative culture of each OTU was subjected to DNA extraction and identification as described below. About 1 g of fresh fungal mycelia was taken and ground in liquid nitrogen. DNA was then extracted using Qiagen DNeasy Plant Kits (Qiagen Inc., Valencia, CA, USA) according to the supplier's protocol. Three different pairs of primers (ITS1F/ ITS4, LR1/ FLR2, EF1/ EF2; Table 3.1) were used for PCR amplification in a 25 µL reaction mix using a 96 well PCR thermocycler machine (Eppendorf AG, Hamburg, Germany). PCR conditions were modified from Mavragani (2008) and Corredor (2011) as follows: 94°C for 3 min, 35 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 10 min for the ITS1F/ ITS4 region. For the Elongation Factor EF1/ EF2 and for LR1/ FLR2, the PCR conditions were: 94°C for 2 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

Table 3.1 Primer, adaptors and key sequences used in this study

Primer	Sequence	Reference
ITS-1F	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns 1993)
ITS2	GCTGCGTTCTTCATCGATGC	(White et al. 1990)
ITS-4	TCCTCCGCTTATTGATATGC	(White et al. 1990)
EF1	ATGGGTAAGGA(A/. G)GACAAGAC-	(O'Donnell et al. 1998)
EF2	GGAAGTACCAGTGATCATGTT	(O'Donnell et al. 1998)
LR1	GCATATCAATAAGCGGAGGA	(van Tuinen et al. 1998)
FLR2	GTCGTTTAAAGCCATTACGTC	(Trouvelot et al. 1999)
Adaptor/ Key (Forward)	CCATCTCATCCCTGCGTGTCTCCGAC/ TCAG	(Roche 2009)
Adaptor/ Key (Reverse)	CCTATCCCCTGTGTGCCTTGGCAGTC/ TCAG	(Roche 2009)

PCR products were run on agarose gels to verify the success of the amplifications and the positive PCR products were submitted to the NRC Plant Biotechnology Institute in Saskatoon for Sanger sequencing. Sequences were edited and compared to NCBI database using Basic Local Alignment Search Tool (BLAST) using default settings. *Fusarium* sequences were also BLASTed against *Fusarium* ID (Geiser et al. 2004) database (<http://board.Fusariumdb.org/board2.php?a=dv&nu=42&id=2>).

3.3.6 Construction of Functional Groups

The definition of fungal functional groups is a practical approach that can provide valuable information in microbial community studies (Zak and Visser 1996). It increases the statistical power by summing up the minor effects on individual species with similar functions which otherwise would remain undetected. The abundance of fungal species with similar functionality was summed up to construct functional groups (Olof et al. 1990). Members of the genera *Trichoderma*, *Bionectria*, *Myrothecium*, and *Nectria*, which are known for including antagonistic species against pathogens (Chatterton et al. 2008; De Schutter et al. 2002; Gülay and Grossmann 1994; Lahlali and Hijri 2010; McQuilken et al. 2001; Rodríguez et al. 2011; Zheng et al. 2011), were placed in the group of fungal antagonists. *Fusarium culmorum*, *Fusarium acuminatum*, *Cochliobolus sativus* and *Gaeumannomyces graminis*, which are known highly virulent pathogens of wheat, were grouped as highly virulent pathogens (Bockus and Shroyer 1998; Esmaeili Taheri et al. 2011; Gonzalez and Trevathan 2000). The mycoparasitic interaction of most fungal antagonists on well known soil borne pathogens was observed in co-culture (Barnett and Lilly 1962; Paul 1999; Prapagdee et al. 2008; Sharma 2011).

3.3.7 Statistical Analysis

Permutation-based MANOVA (perMANOVA) statistical analysis was used to test the significance of fungicide effect on fungal community structure on both, plate culture and pyrosequencing data using the package Vegan (Oksanen et al. 2011) in R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria) (2011). Non-metric multidimensional scaling (NMDS) was applied to explore the effects of year, root health and fungicide treatment on the fungal communities and the relationships between fungal taxa using PC-ORD statistical program

(MjM Software, Gleneden Beach, OR, USA) (McCune and Mefford 2011). Fungi with less than 2% of total number of isolates were excluded from the NMDS analysis of the plate culture data (McCune et al. 2002). In NMDS plots, centroid ovalays of independent variables displayed as a plus sign are provided in the ordination space using PC-ORD (MjM Software, Gleneden Beach, OR, USA) (McCune and Mefford 2011). The distance between the centroid of a variable with specific fungal taxon is a measure of abundance of the target fungus in relation to that variable (the shorter the distance between them means that the target fungal taxon was more abundant where that specific variable was in place). Pyrosequencing data were relativized by row total (McCune et al. 2002). The 454 pyrosequencing reads making up less than 1% of all reads obtained were excluded from the analysis (McCune et al. 2002). The significance of fungicide effect on the abundance of the functional groups of fungi was tested by ANOVA using the mixed model in SAS/STAT software, version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) (1999). Arcsine square root and rank transformation was used to improve normality. Ls-Means were grouped using Macro PDMIX 800 (Saxton 1998) based on Least Significant Difference (LSD). Spearman correlation was computed to look at the correlation between durum wheat yield and the abundance of different functional groups or fungal taxa using R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria) (2011).

3.4 Results

3.4.1 Pyrosequencing

Pyrosequencing revealed more fungal genera in the root mycota than in plate culture (data not shown). The genera identified by pyrosequencing belonged to five phyla of the fungal Kingdom and Ascomycota was the most abundant phylum (Figure 3.1). *Fusarium* was the predominant genus in the roots of durum wheat at the heading stage based on pyrosequencing in both years (2009 and 2010), especially in 2009, which was a relatively dry year (Figures 3.2 – 3.3). The prevalence of *Fusarium* was clearly reduced in 2010 a record wet year (Figures 3.2 – 3.3).

Olpidium, a soil-borne root infecting pathogen (Hartwright et al. 2010) and a vector of plant viruses (Campbell 1996), yet unreported from durum wheat before (Farr and Rossman

2010) was revealed by 454 pyrosequencing as a major component of durum root mycota in 2010 an extremely wet year but not in 2009 (Figures 3.2 – 3.3). The pyrosequencing data showed a significant three-way interaction of fungicide, year, and root type (Table 3.2). NMDS ordination revealed that the fungicide use on chickpea in 2008 favored the proliferation of the genus *Fusarium* in durum wheat roots in 2009 but proliferation of *Fusarium* in durum roots in 2010 was not favored by fungicide application on chickpea in 2009 (Figures 3.4-3.5). According to the mixed model analysis of pyrosequencing data, there was a significant interaction of year, fungicide treatment, and root type on the abundance of *Fusarium*. The abundance of this genus was higher in seminal roots after fungicide application in 2009 (Table 3.3, Figure 3.6). Environmental changes in 2009 vs. 2010 were a major factor in shaping fungal community (Figures 3.2-3.3). For instance, while *Olpidium* was rare in 2009 (about 1%), it became the second most predominant taxa in 2010 (about 22%) (Figures 3.2 – 3.3).

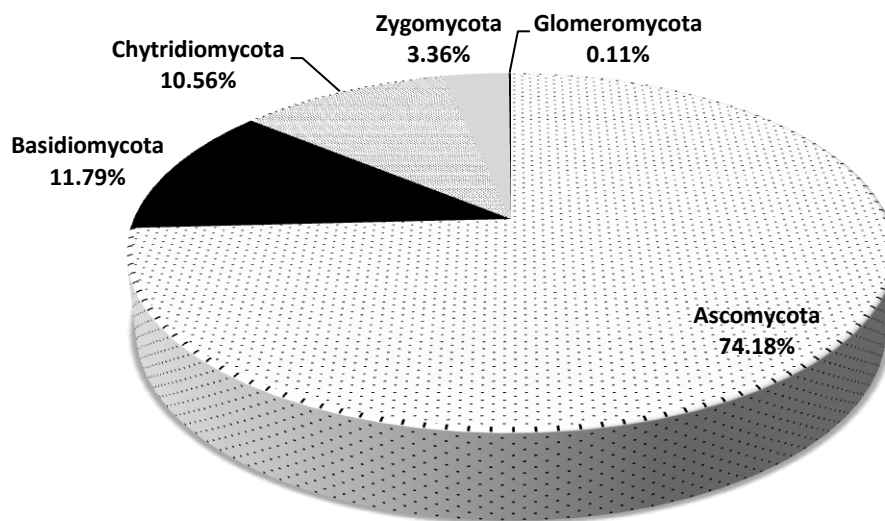


Figure 3.1 Relative abundance of fungi in 2009 and 2010 by phylum level according to pyrosequencing read number N (the total number of plots) = 32.

Table 3.2 Significance of the effect of fungicide treatment, root type, year and their interactions on the structure of the fungal community in durum roots at heading stage following chickpea CDC Vanguard, according the analysis of pyrosequencing data by perMANOVA.

Effect	DF	F Value	P Value
Fungicide treatment	1	2.4745	0.009
Root type	1	3.5183	0.001
Year	1	7.3477	0.001
Fungicide treatment X Root type	1	2.8415	0.004
Fungicide treatment X Year	1	1.5602	0.077
Root type X Year	1	3.0399	0.001
Fungicide treatment X Root type X Year	1	2.5586	0.004

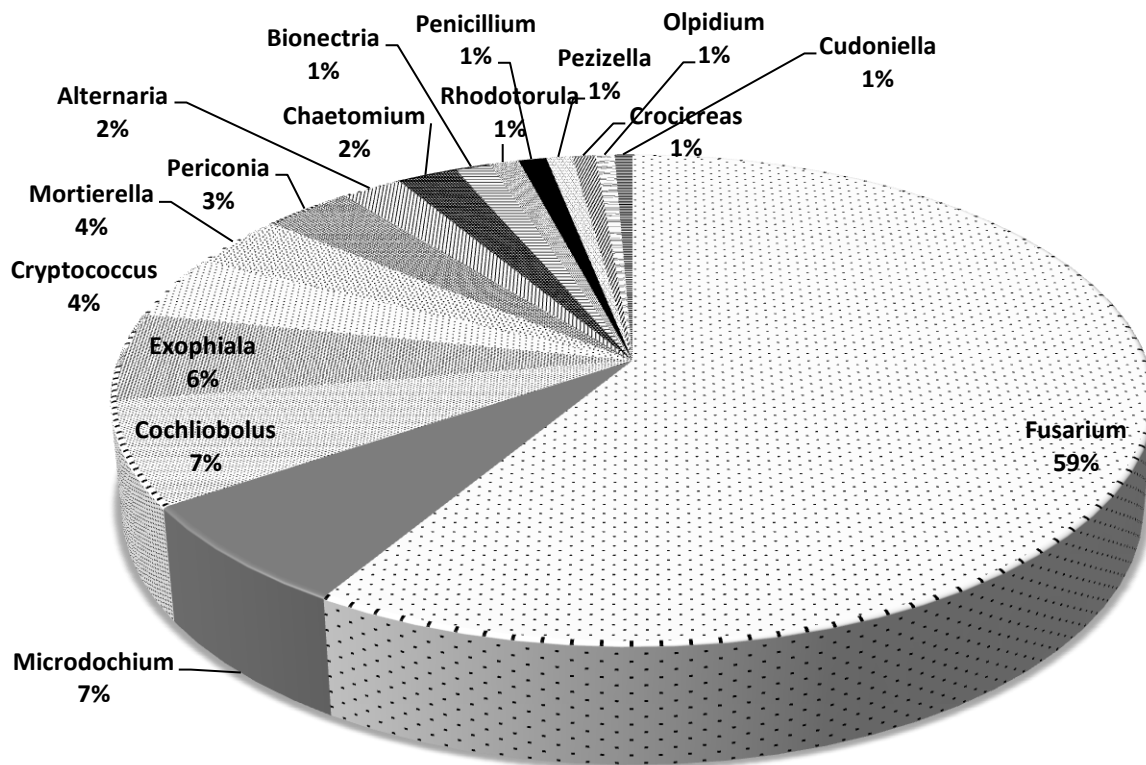


Figure 3.2 Relative abundance of the main fungal genera represented in the roots of durum wheat as revealed by pyrosequencing in 2009. *N*(the total number of plots) = 16, unclassified reads are excluded.

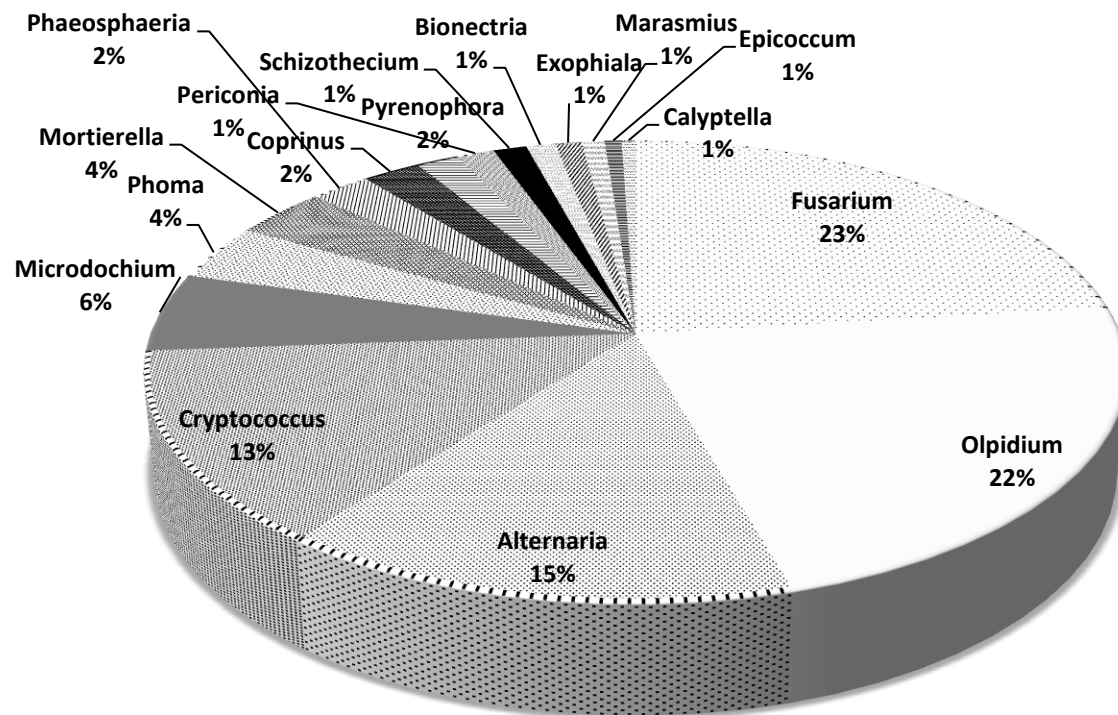


Figure 3.3 Relative abundance of fungal genera in the roots of durum wheat detected by pyrosequencing in 2010. N (the total number of plots) = 16, unclassified reads are excluded.

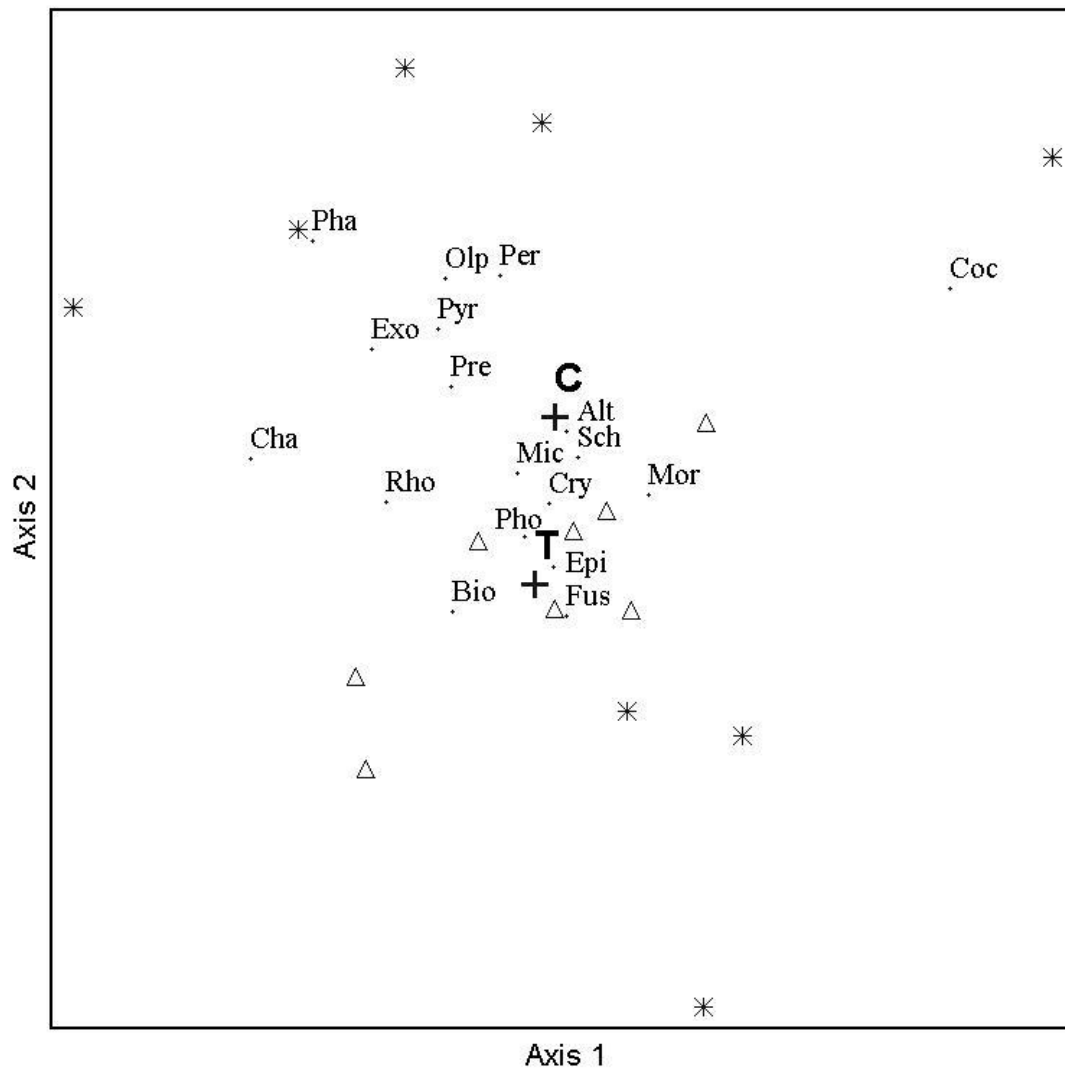


Figure 3.4 NMDS biplot of the fungal community profile of the root endosphere of a durum wheat crop grown in 2009 and sampled at the early heading stage following fungicide treated (triangles) and untreated (stars) chickpea crops, as determined by pyrosequencing. Stress = 10.072, N (the total number of plots) = 16, $P = 0.02$, C = control, T = fungicide treated. Variance explained by axis 1 = 0.293 and by axis 2 = 0.610. Fus = *Fusarium*, Cry = *Cryptococcus*, Alt = *Alternaria*, Olp = *Olpidium*, Mic = *Microdochium*, Mor = *Mortierella*, Exo = *Exophiala*, Per = *Periconia*, Pha = *Phaeosphaeria*, Bio = *Bionectria*, Coc = *Cochliobolus*, Pyr = *Pyrenophora*, Pho = *Phoma*, Sch = *Schizothecium*, Pre = *Preussia*, Rho = *Rhodotorula*, Cha = *Chaetomium*, Epi = *Epicoccum*.

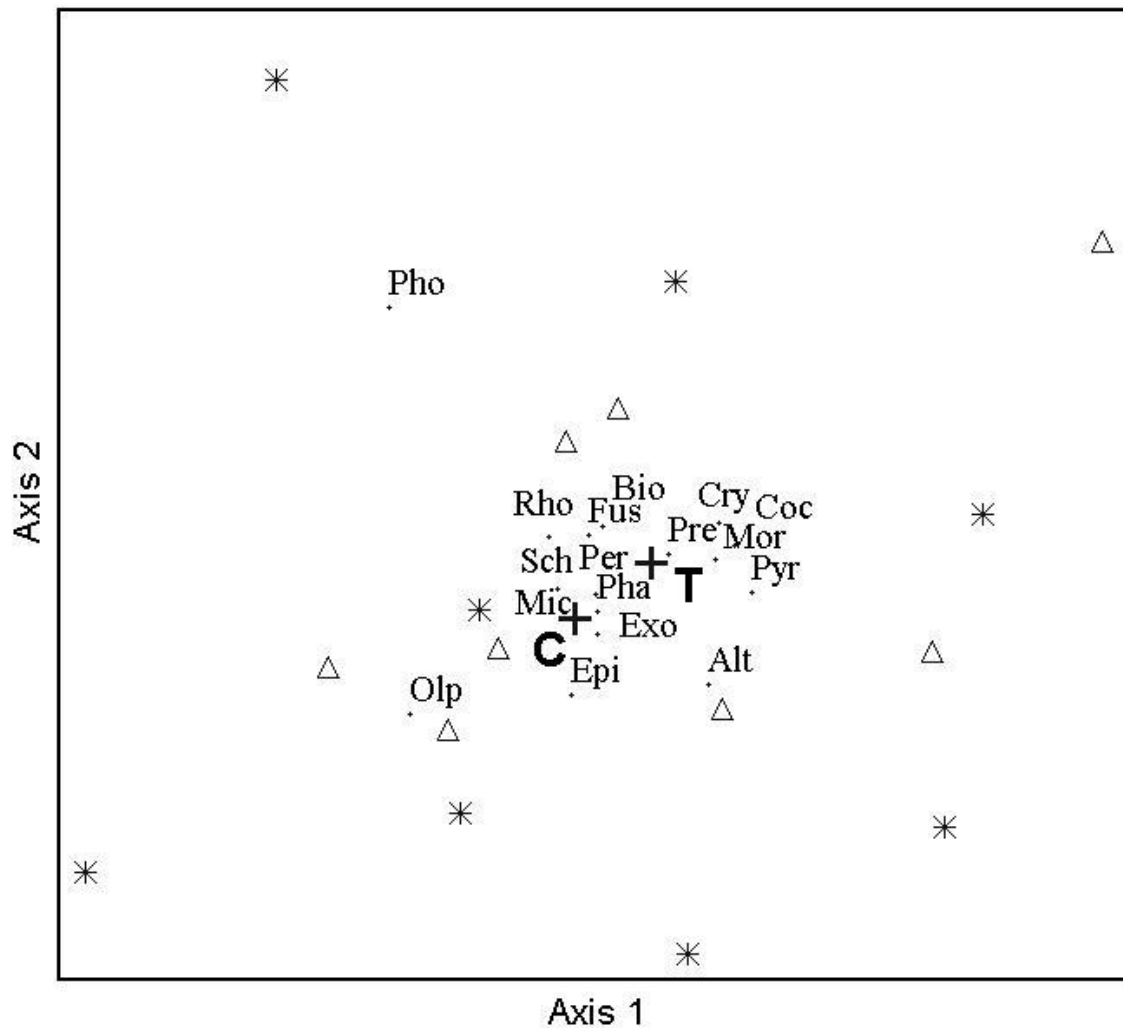


Figure 3.5 NMDS plot of the fungal community profile of the root endosphere of a durum wheat crop grown in 2010 and sampled at the early heading stage following fungicide treated (triangles) and untreated (stars) chickpea crops, as determined by pyrosequencing. Stress = 14.460, N (the total number of plots) = 16, $P = 0.04$. C = control, T = fungicide treated. Variance explained by axis 1 = 0.216 and by axis 2 = 0.610. Fus = *Fusarium*, Cry = *Cryptococcus*, Alt = *Alternaria*, Olp = *Olpidium*, Mic = *Microdochium*, Mor = *Mortierella*, Exo = *Exophiala*, Per = *Periconia*, Pha = *Phaeosphaeria*, Bio = *Bionectria*, Coc = *Cochliobolus*, Pyr = *Pyrenophora*, Pho = *Phoma*, Sch = *Schizothecium*, Pre = *Preussia*, Rho = *Rhodotorula*, Cha = *Chaetomium*, Epi = *Epicoccum*.

Table 3.3 Significance of the effect of fungicide treatment, root type, year and the interactions of the factors on the relative abundance of 454 pyrosequencing reads of *Fusarium* in durum roots at heading, according to mixed model analysis of pyrosequencing data.

Effect	DF	F Value	P Value
Year	1	11.44	0.0011
Fungicide treatment	1	2.25	0.2903
Root type	1	6.16	0.0083
Year X Fungicide treatment	1	2.74	0.3616
Year X Root type	1	0.13	0.5254
Fungicide treatment X Root type	1	1.41	0.0318
Year x Fungicide treatment X Root type	1	4.71	0.0127

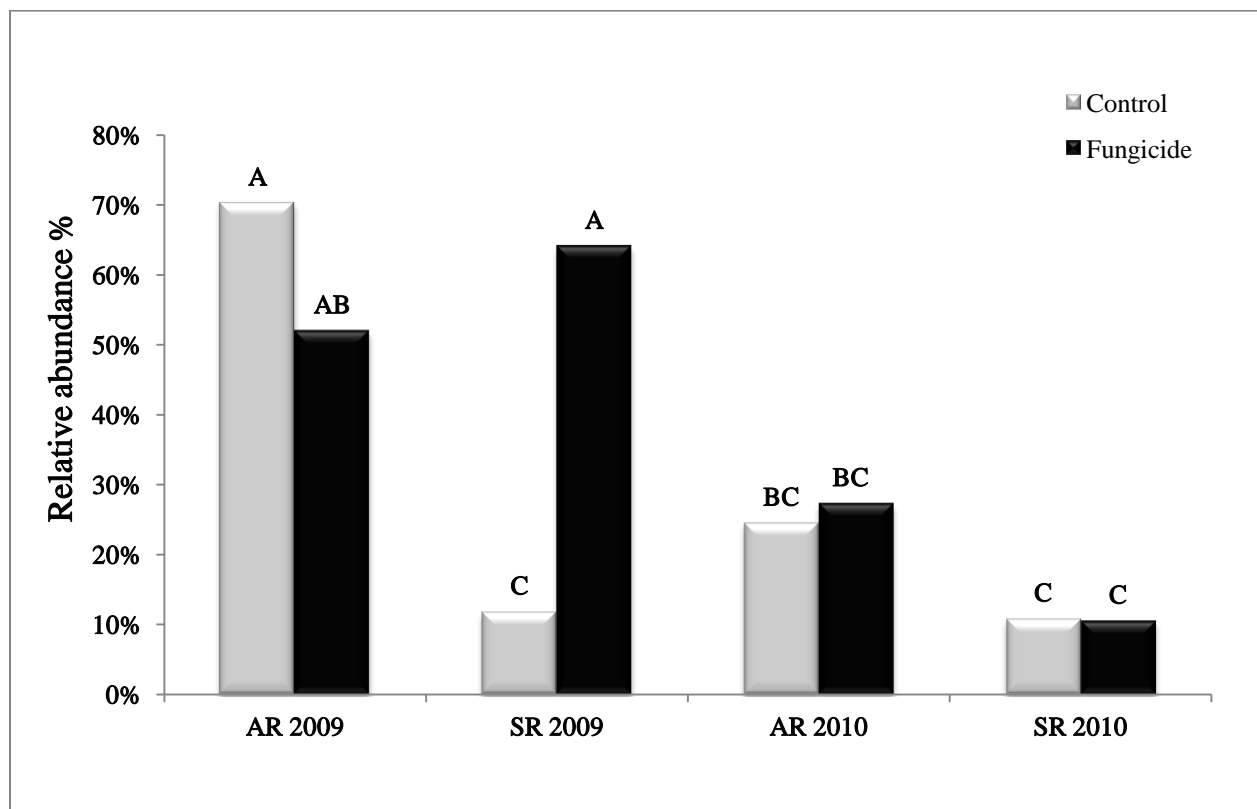


Figure 3.6 The effect of fungicide treatments on chickpea on the relative abundance of the genus *Fusarium* in seminal roots (SR) and adventitious roots (AR) of subsequent durum wheat at early heading as determined by pyrosequencing, $P = 0.012$, $n = 4$.

3.4.2 Plate Culture

The communities of culturable fungi associated with healthy and diseased roots of durum wheat were unaffected by fungicide application on chickpea, but there was a significant interaction between root health status and year on fungal community (Table 3.4). According to NMDS analysis, the majority of fungal species associated with healthy roots were dark septate endophytes (DSE) while these fungi were rare in symptomatic roots (Figure 3.7). Fungal species were differently distributed in 2009 vs. 2010 (Figure 3.8).

Table 3.4 The effect of fungicide treatment, chickpea cultivar, root health status and year on overall culturable fungal community of durum root following chickpea CDC Luna and CDC Vanguard at maturity stage in 2009 and 2010 according to perMANOVA analysis.

Source	Df	F Value	P Value
Fungicide treatment	1	0.01052	0.343
Chickpea cultivar	1	0.00680	0.668
Year	1	0.13866	0.001
Root health status*	1	0.16862	0.001
Fungicide treatment X Chickpea cultivar	1	0.00455	0.870
Fungicide treatment X Year	1	0.01950	0.061
Chickpea Cultivar X Year	1	0.00750	0.607
Fungicide treatment X Root health status	1	0.01146	0.305
Chickpea Cultivar X Root health status	1	0.00757	0.625
Year X Root health status	1	0.12593	0.001
Fungicide treatment X Chickpea cultivar X Year	1	0.01091	0.321
Fungicide treatment X Chickpea cultivar X Root health status	1	0.00894	0.476
Fungicide treatment X Year X Root health status	1	0.00802	0.556
Chickpea cultivar X Year X Root health status	1	0.00745	0.591
Fungicide Treatment X Chickpea cultivar X Year X Root health status	1	0.00841	0.534

*Fungal communities associated with healthy and diseases roots were isolated separately to see the effect of fungicides on endophytes and pathogens precisely.

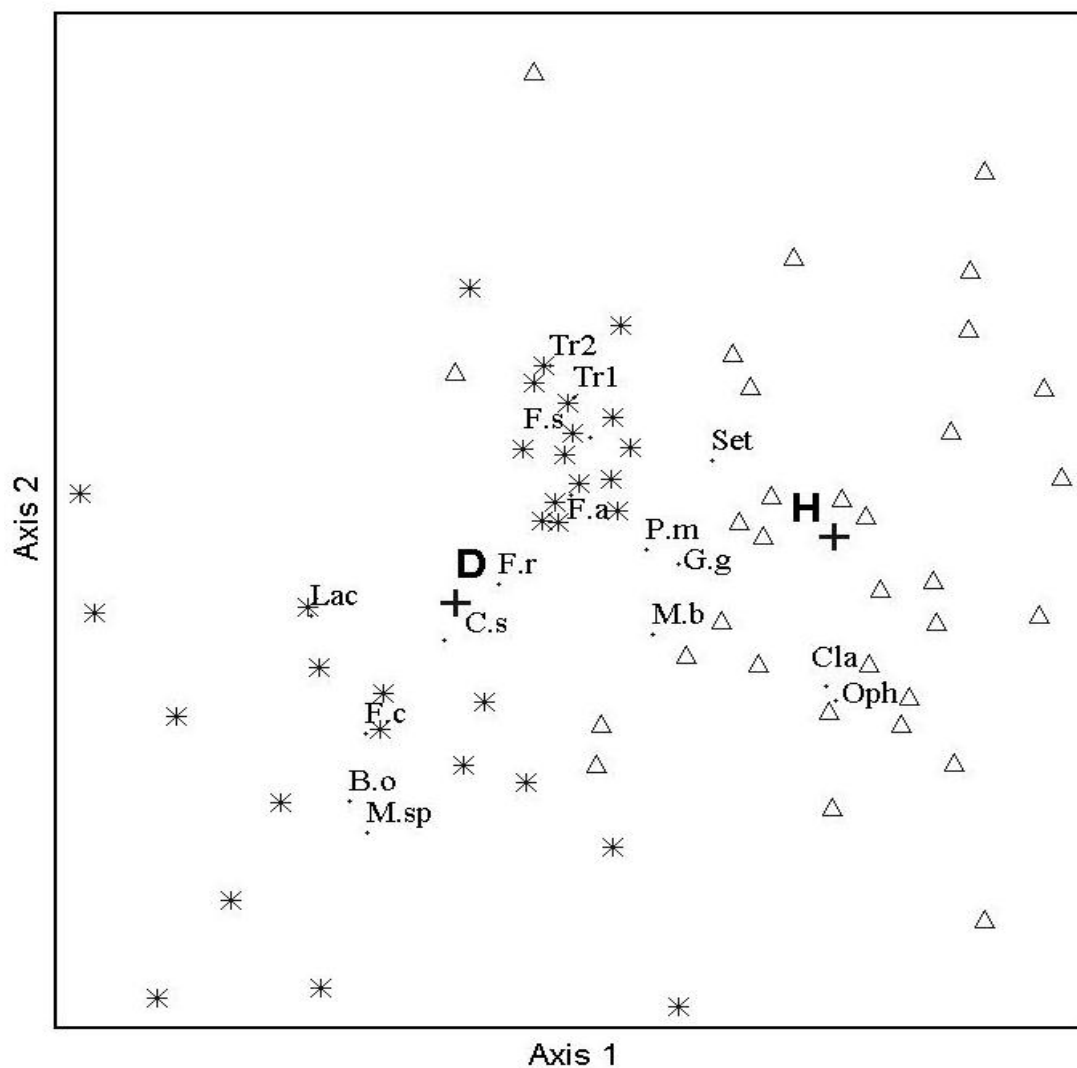


Figure 3.7 NMDS plot of the fungal community profile of the healthy (triangles) and diseased (stars) root endosphere of a durum wheat crop sampled at the maturity stage determined by plate culture. Stress = 19.6, N (the total number of plots) = 64, $P = 0.02$, H = healthy roots, D = diseased roots. Variance explained by axis 1 = 0.189 and by axis 2 = 0.605. C.s = *Cochliobolus sativus*, F.r = *Fusarium redolens*, F.c = *Fusarium culmorum*, G.g = *Gaeumannomyces graminis*, P.m = *Periconia macrospinosa*, F.s = *Fusarium solani*, Set = *Setophoma* sp., M.sp = *Myrothecium* sp, B.o = *Bionectria* sp, Lac = *Lachnella* sp., F.a = *Fusarium acuminatum*, Tr1 = *Trichoderma* (10-3), Tr2 = *Trichoderma* (10-17), M. b = *Microdochium bolleyi*, Cla = *Cladosporium* sp., Oph = *Ophiosphaerella* sp..

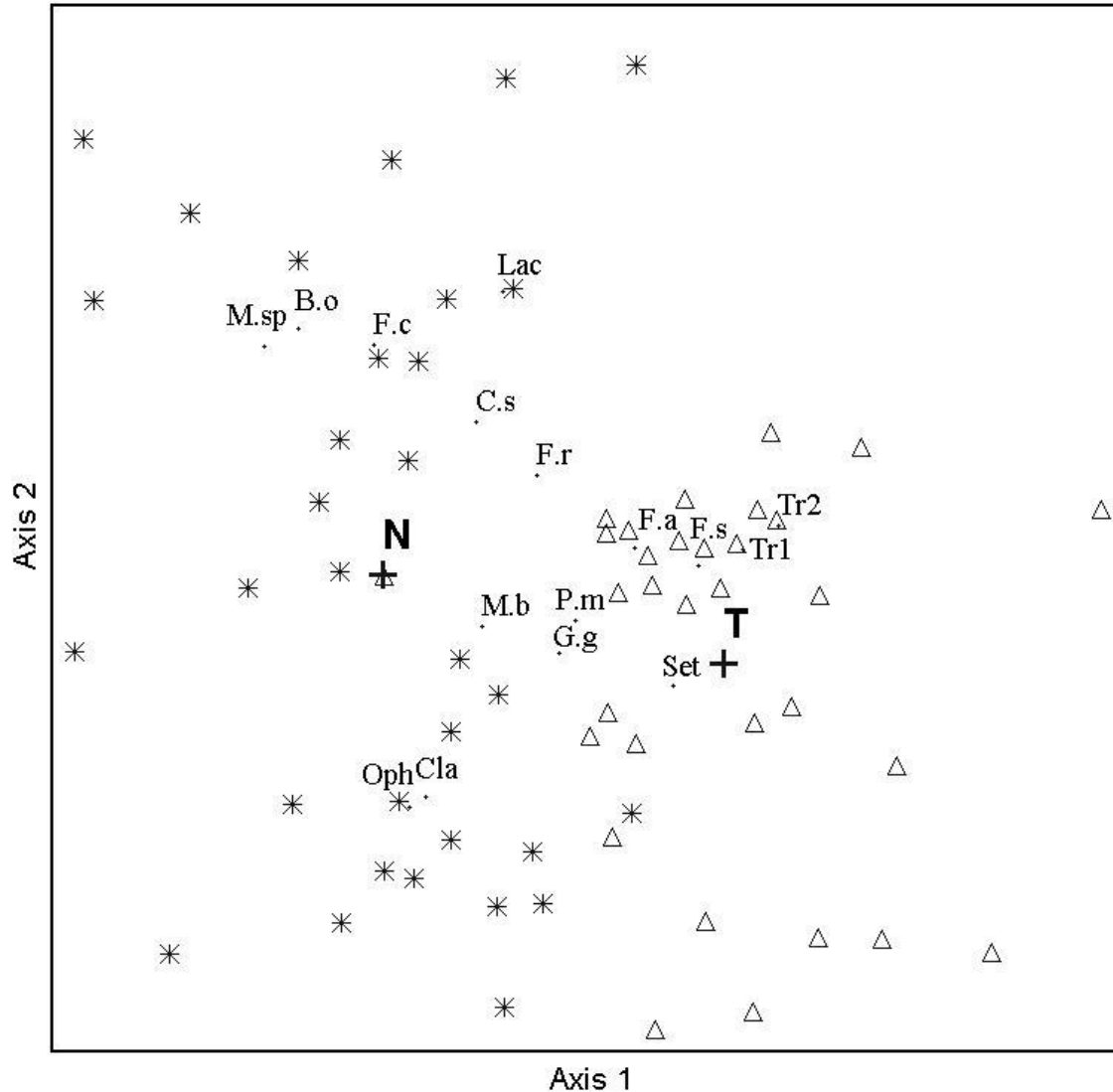


Figure 3.8 NMDS plot of the fungal community profile of root endosphere of a durum crop sampled at the maturity stage in 2009 (stars) and 2010 (triangles) as determined by plate culture. Stress = 19.6, N (the total number of plots) = 64, $P = 0.02$, $N = 2009$, $T = 2010$. Variance explained by axis 1 = 0.189 and by axis 2 = 0.605. C.s = *Cochliobolus sativus*, F.r = *Fusarium redolens*, F.c = *Fusarium culmorum*, G.g = *Gaeumannomyces graminis*, P.m = *Periconia macrospinos*, F.s = *Fusarium solani*, Set = *Setophoma* sp., My.sp = *Myrothecium* sp., B.o = *Bionectria* sp., Lachnel = *Lachnella* sp., F.a = *Fusarium acuminatum*, Tr1 = *Trichoderma* (10-3), Tr2 = *Trichoderma* (10-17), M. b = *Microdochium bolleyi*, Cla = *Cladosporium* sp., Oph = *Ophiosphaerella* sp..

The abundance of culturable antagonists in symptomatic roots was significantly decreased by fungicide application (Table 3.5, Figure 3.9); whereas, the abundance of the highly virulent pathogens was not affected (Figure 3.9). The abundance of total fungi was not affected by fungicide (Figure 3.9). There were more fungal antagonists in symptomatic durum wheat roots after chickpea CDC Luna than CDC Vanguard (Table 3.5, Figure 3.10).

Table 3.5 Significance of the effect of fungicide treatment, chickpea cultivar, year, and their interactions on the abundance of culturable fungal antagonists of symptomatic durum root at the maturity stage, according to mixed model analysis.

Effect	DF	F Value	P Value
Year	1	3.46	0.0874
Chickpea cultivar	1	7.41	0.0345
Fungicide treatment	1	7.93	0.0156
Year * Chickpea cultivar	1	0.12	0.7331
Year * Fungicide treatment	1	1.10	0.3157
Chickpea cultivar * Fungicide treatment	1	1.25	0.2859
Year * Chickpea cultivar * Fungicide treatment	1	1.25	0.2859

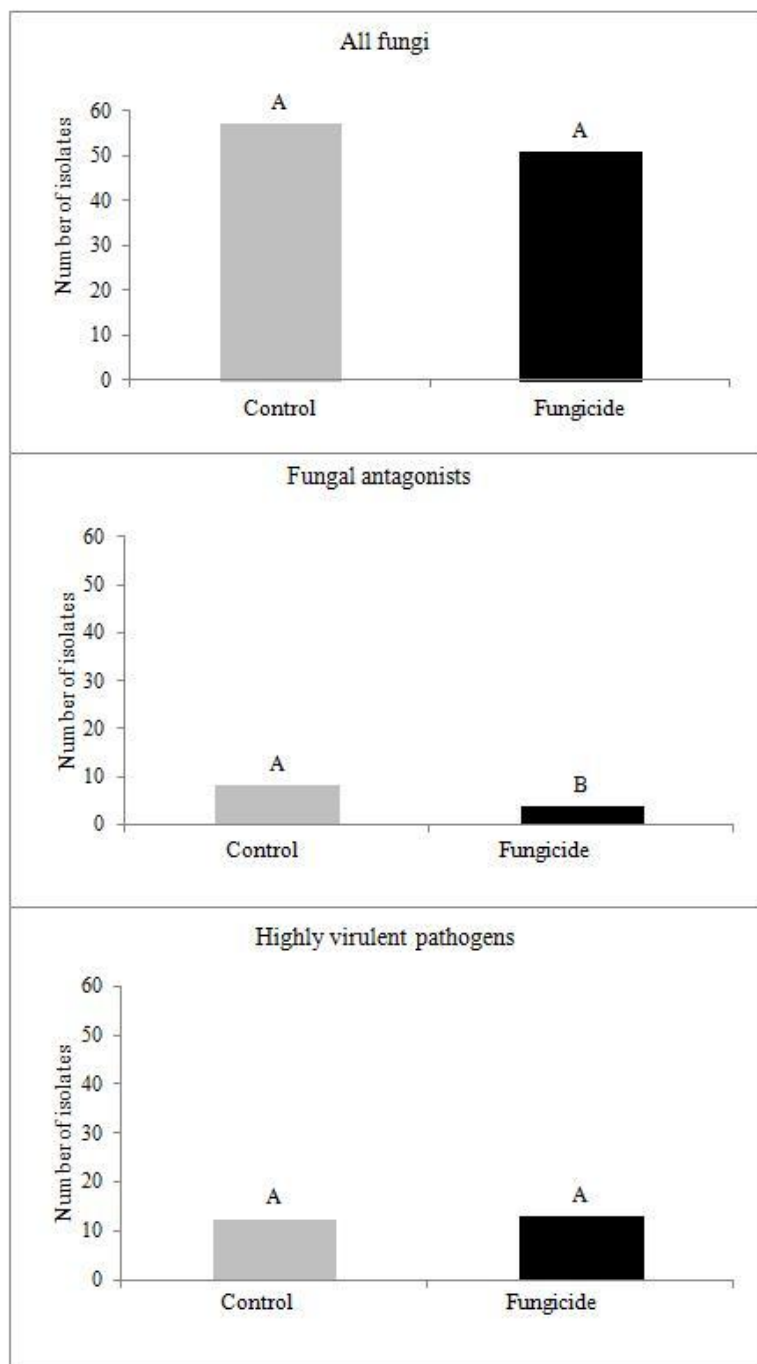


Figure 3.9 Abundance of total culturable fungi, fungal antagonists and highly virulent pathogens in symptomatic roots of durum wheat at maturity as influenced by fungicide application on a previous crop of chickpea (CDC Vanguard and CDC Luna). In each group, bars with different letters are significantly different at $P < 0.05$; $n = 16$. Highly virulent pathogens = *C. sativus*, *G. graminis*, *F. culmorum*, *F. acuminatum*, Fungal antagonists = *Trichoderma* spp., *Myrothecium* sp., *Nectria* sp. and *Bionectria* sp.

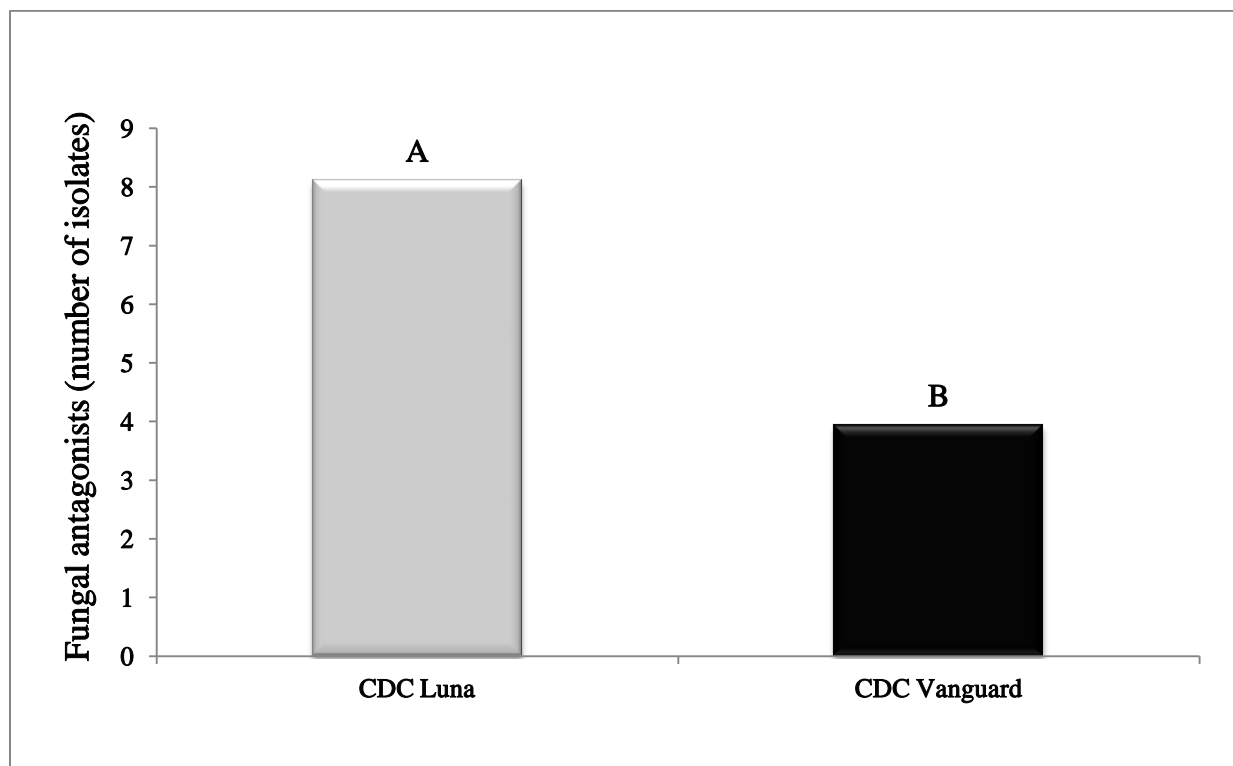


Figure 3.10 Effect of chickpea genotype on the abundance of culturable fungal antagonists (*Trichoderma* spp., *Myrothecium* sp., *Nectria* sp. and *Bionectria* sp.) in the diseased roots of durum wheat at maturity following in rotation, in 2009 and 2010, bars with different letters are significantly different at $P < 0.05$, $n = 16$.

3.5 Discussion

Fusarium spp. in the roots of durum wheat in 2009 were associated with fungicide use on chickpea in 2008, as revealed by pyrosequencing. This genus includes many devastating soil-borne pathogens such as *F. culmorum*, *F. acuminatum*, *F. graminearum*, and *F. avenaceum* (e.g. Bockus and Shroyer 1998; Gonzalez and Trevathan 2000), therefore it may have a negative effect on plant productivity. Pyrosequencing analysis also showed that *Fusarium* was predominant in the roots of durum wheat at the early heading stage suggesting its relevance in plant health and productivity. However, fungicide use on chickpea did not affect durum yield significantly the following year, which suggests a limited effect of increased population of *Fusarium* on yield. Lupwayi and Kennedy (2007) have suggested that fungicides applied on

pulses may suppress pathogens in subsequent crops. This is not supported, at least for some pathogens, by the current study. Kirkegaard et al. (2004) reported a relationship between wheat yield and severity of disease caused by *F. pseudograminearum* after different rotation crops, which generally agrees with the results of the current study. Some root pathogens of wheat can survive in the stubble of the previous year's crop (Bockus and Shroyer 1998; Fernandez and Fernandes 1990). Reduced degradation of stubble may thus favor the survival of the pathogens and increase disease incidence (Fernandez and Fernandes 1990). Fungicide application may improve the chance of survival of pathogens by suppressing the saprotrophic fungi (Robinson et al. 2005) that degrade stubble. Chlorothalonil and pyraclostrobin particularly affect spores germination (Balba 2007; Bartlett et al. 2002). Since fast spore germination is a main physiological characteristic of some saprotrophs (Garrett 1951), this group of fungi may be affected more by fungicide contamination of the soil compared to root-inhabiting pathogens (Robinson et al. 2005). It is shown that retaining the previous year stubble could increase the incidence of soil-borne disease of wheat; whereas, removing or burning them could decrease it (Burgess et al. 1993; Summerell et al. 1989; Wildermuth et al. 1997) which supports the above-mentioned hypothesis.

Systemic Acquired Resistance (SAR) may also explain lower populations of *Fusarium* in untreated plots. SAR could be induced in chickpea after ascochyta infection of aerial plant parts, therefore the roots of ascochyta-infected chickpea in control plots may become more resistant to soil-borne pathogens thereby causing reduction in pathogen populations in soil in the following spring (Ryals et al. 1994). In the same experiment as the current study, Yang et al. (2012c) have shown that the chemical control of ascochyta blight was very effective in 2008 when disease pressure was high but it was useless in 2009 when disease pressure was minimal due to dry weather. In the other words, unlike in 2009, in the year 2008 the higher disease severity in control plots may have resulted in increased activation of SAR system and lower root disease infection. Consequently the population of soil borne pathogens in control versus treated chickpea plots might have been different in 2008 but not in 2009. This is in agreement with the results of the current study in the plots of durum wheat of following years (2009 and 2010 respectively) where the population of *Fusarium* spp. in the seminal roots was increased by fungicide in 2009 and not in 2010. Results of Cruz et al. (2012) also supports the above mentioned hypothesis.

They showed a decrease in the amount of volatile organic compounds with suppressive effect on pathogenic *Fusarium* spp. in chickpea root due to the control of ascochyta blight with fungicide (Cruz et al. 2012; Penuelas and Llusà 2001)

Increase in the population of *Fusarium* spp. due to fungicide application might raise concern about the likelihood of *Fusarium* root and crown diseases outbreaks due to long-term fungicide application. The effect of fungicide use on previous-year chickpea on the fungal communities of root endosphere of durum was less profound in 2010, a record wet year, which suggests the likely role of moisture in diminishing non-target effects of fungicide residues through increased sorption, degradation or fungicide leach-out (Roy et al. 2000). The other likely explanation for stronger effect of fungicides applied in 2008 (on chickpea) compared with fungicides applied in 2009 could be the fact that the precipitation in 2008 was more than 2009. More precipitation in 2008 could result in washing more fungicides from plant leaves and adding them to the soil which means a more profound impact on soil fungal communities.

According to plate culture, the abundance of antagonists in symptomatic roots of durum wheat was suppressed by fungicide use on chickpea, but the group of highly virulent pathogens and total fungi remained unaffected. The negative effect of fungicide on antagonists was predictable but it is interesting that highly virulent pathogens did not follow a similar pattern. Lower stubble degradation rate in fungicide-treated chickpea plot or SAR, as explained earlier, may be a reason for unaffected populations of pathogens due to fungicide (please see: Bockus and Shroyer 1998; Cruz et al. 2012; Penuelas and Llusà 2001; Ryals et al. 1994).

Root and crown diseases of wheat are a major problem in wheat production worldwide (Cook 2007; Ledingham et al. 1973; Smiley et al. 2005; Wildermuth et al. 1992). Reduced populations of indigenous fungal antagonists, including species of *Trichoderma*, *Myrothecium*, *Nectria*, and *Bionectria* (Chatterton et al. 2008; De Schutter et al. 2002; Gülay and Grossmann 1994; Lahlali and Hijri 2010; McQuilken et al. 2001; Rodríguez et al. 2011; Zheng et al. 2011) which were negatively correlated to known pathogens (thought not statistically significant), may partly-explain the poor performance of durum wheat after chickpea. A positive effect of fungal antagonism in crown disease control and yield is suggested by Kirkegaard et al. (2004) which is consistent with trends observed in the current study. However, while I observed a negative

correlation of durum yield with the population of known pathogens and a positive correlation of yield with the abundance of natural antagonists, which is fairly consistent with my initial hypothesis, none of these correlations was statistically significant which may be due to insufficient replicate numbers.

The cultivar CDC Luna, a Kabuli chickpea (large-seeded) (Nayyar et al. 2006), promoted the proliferation of culturable fungal antagonists more than the cultivar CDC Vanguard, a Desi chickpea (small-seeded) (Nayyar et al. 2006). Current results suggest an influence of plant genotype on the structure of associated microbial communities, which supports the results of previous studies (Andreote et al. 2010; Xu et al. 2009). Gan et al. (2007) have shown that Kabuli cultivars were more susceptible to ascochyta blight than Desi cultivars. The effect of chickpea cultivar on associate microbes observed here and reported by Gan et al. (2007) could be due to different phytochemical profiles of each cultivar (Cruz et al. 2012). For instance volatile compounds, a group of phytochemicals with likely antimicrobial activities, could be different in chickpea cultivars as shown by Cruz et al. (2012). The current finding might be valuable for selecting appropriate cultivars in cropping systems. Also breeders may study the genetic basis of difference in beneficial microbe enhancement efficiency of CDC Luna and CDC Vanguard in the search for useful traits. More research is required to explain the mechanisms causing differences in the abundance of fungal antagonists after CDC Luna and CDC Vanguard.

There was no effect of fungicide on the community of culturable fungi in the roots of mature durum wheat. Many studies looking at the effects of fungicides on microbial community composition under field conditions detected minor, or no, effects on microbial communities even when the effect of fungicides were monitored during the same growing season as fungicide application (Girvan et al. 2004; Sigler and Turco 2002). Degradation of fungicide residuals (Potter et al. 2001) during one year interval between fungicide application and the fungal isolation, as well as exclusion of unculturable fungi from the analysis due to the limitation of culture-dependent methods (Kirk et al. 2004; Torsvik et al. 1998) may have compromised my ability to detect likely effects at the community level. Out of three active ingredients applied, boscalid was the only one detected in chickpea rhizosphere few weeks after the last spray (Yang et al. 2012b), which supports this speculation. Higher persistence of this ingredient in the soil

compared with pyraclostrobin and chlorothalonil (Lagunas-Allué et al. 2010; Potter et al. 2001; USEPA 2003; Zhang et al. 2012) may cause more non-target effects on the soil microbes. This suggests the need for in-depth investigation about degradation procedure and effects of boscalid on the important members of the community this may result in advocating the substitution of it with a less-persistent compound. More studies on the effect of active ingredients used in this study on the growth and reproduction of functionally-important fungi are warranted.

Isolates of fungi obtained from plate culture were grouped into OTU based on careful morphology examination. This is a practical approach to reduce the number of subsequent PCR reactions but could be associated with some errors. Grouping fungal species with similar function in functional groups is recommended and used for soil biological process studies (Olof et al. 1990). Zak and Visser (1996) have suggested that looking only at biodiversity data without attempting to link taxonomic diversity with functional diversity yields little predictivity, and Bengtsson (1998) suggested to pay more attention to understand the relation of functional groups and ecosystem functions rather than species diversity *per se*. Regardless of the mechanism involved, change in the proportion of different fungal functional groups in relation to fungicide application may be worth noting in evaluating the effect of fungicides on the functionality of system. More research about the effects of fungicides on ecological fitness of functionally-important fungi and their relation with plant health status and yield is needed.

Despite limitations like short sequences (Haas et al. 2011), pyrosequencing seems to be a reliable quantitative method for monitoring microbial community structure at the field level. This method detected more biodiversity at the genus level compared with plate culture. For example, the genus *Olpidium*, a soil borne plant pathogen (Hartwright et al. 2010) and a known vector of plant viruses (Campbell 1996), was not isolated from durum roots (Farr and Rossman 2010) using plate culture but pyrosequencing revealed that it was the predominant component of the root mycota in 2010. This may explain why unlike plate culture, some important effects were detected on root fungal community by pyrosequencing.

3.6 Conclusion

Fusarium, the predominant taxon in the roots of durum wheat at the early heading stage, was associated with fungicide application on chickpea conducted during the previous year according to pyrosequencing. Although no significant effect of chickpea-targeted fungicide on the yield of durum in the following year was detected, a negative correlation between relative abundance of this genus with durum yield may provide a partial explanation for the poor performance of durum wheat after chickpea. The plate culture results suggest that while fungicide treatment may not affect the entire community, a specific functional group may be affected significantly, which could compromise the functionality and productivity of the system. This supports the suggestions made by Bengtsson (1998) who indicated that more attention needs to be paid to the functional diversity of species rather than biological diversity *per se*. Chickpea genotype is an important factor in shaping the community of functionally important fungi in the soil. As expected, pyrosequencing detected a wider spectrum of fungal community in the root system compared with plate culture, and proved to have higher resolution in detecting treatment effect.

3.7 Connection to the Next Study

Foliar fungicide application suppressed the abundance of culturable fungal antagonists, but did not affect or increased the population of potentially-highly virulent pathogens. To reveal whether differences in the reaction of fungal functional groups is a result of a direct effect on growth, in the next chapter, I examined the effect of fungicide on fungal growth *in vitro*.

4. FUNGICIDE TOLERANCE OF IMPORTANT FUNGI ISOLATED FROM DURUM WHEAT ROOTS

4.1 Abstract

The toxicity of fungicides commonly used to control ascochyta blight in chickpea on most-abundant or functionally-important fungi was tested *in vitro*. The goal was to determine whether the pattern of fungicide-driven changes in fungal abundance in the field (chapter three) could be due to direct growth inhibition of fungicides or whether other mechanism(s) were involved. Two fungicide treatments were used: 1) a mixture of three fungicides, Bravo, Headline and Lance, at equal active ingredient concentration, and 2) Lance alone. Two fungicide combinations used in this study affected the growth of fungal species differently and some fungi were more sensitive to a specific fungicide combination. For instance, the mixture of three fungicides suppressed *Trichoderma* OTU 10-17, *Gaeumannomyces graminis*, *Cochliobolus sativus* and *Fusarium culmorum* the most, while *F. redolens* and *F. acuminatum* were the least-sensitive species. On the other hand Lance inhibited the growth of most fungi, but had no effect on some pathogenic species, including *G. graminis* and *F. acuminatum*. The response of highly virulent pathogens and fungal antagonists to the *in vitro* fungicides treatments was similar, in contrast to the results of the field study which suggested that the effect of fungicides on fungal community was indirect.

4.2 Introduction

Fungicides have different modes of action and target different metabolic pathways to inhibit the growth and reproduction of fungi. Fungi are diverse, therefore a range of fungicide tolerance may exist in agro-ecosystems (Avenot et al. 2008). Some fungi have alternative pathways that help them escape the effect of certain fungicides (Wise et al. 2008). Fungicide resistance may also develop in susceptible fungi through mutation and selection in fungicide-affected environments (Avenot et al. 2008). Many studies are done on the effect of different fungicides on a specific fungal species or a certain fungal functional group (Chiocchio et al. 2000; Diedhiou et al. 2004; Kopacki and Wagner 2006; Zambonelli and Iotti 2001). However controlled condition experiments are rarely used as a complementation to the effect of fungicides in the environment. I observed different responses to foliar fungicide use on chickpea on different fungal species and functional groups (chapter three). While culturable fungal antagonists were suppressed by fungicides, highly virulent root pathogens were not impacted. I

hypothesized that different responses to fungicides were due to the different fungicide tolerance of various fungal species. This study examined the fungicide tolerance of important soil-borne fungi isolated from the roots of field-grown durum wheat plants. The goal was to determine if the field level effect of fungicide on different functional groups can be the result of a direct growth inhibition. The knowledge of the impact of fungicidal products on important fungi may help us to select fungicides with minimal negative impact on potentially beneficial members of the fungal community.

4.3 Materials and Methods

4.3.1 Experimental Design

Two experiments were done in complete randomized design with three replicates. Sensitivity of twelve fungal isolates, including *Fusarium redolens* (OTUs 18 and 27), *F. culmorum*, *F. acuminatum*, *Gaeumannomyces graminis*, *Cochliobolus sativus*, *Trichoderma* OTU 10-3, *Trichoderma* OTU 10-17, *Myrothecium* sp, *Bionectria* sp, *Setophoma* sp, and *Microdochium bolleyi*, to two different fungicide treatments was assessed. Fungicide treatments were: 1) Lance (a.i. boscalid, BASF Canada Inc., Mississauga, ON) at 100 ppm, and 2) a mixture of Lance (a.i. boscalid, BASF Canada Inc., Mississauga, ON), Headline (a.i. pyraclostrobin BASF Canada Inc., Mississauga, ON) and Bravo (a.i. chlorothalonil, Syngenta Crop Protection Canada Inc., Guelph, ON) each at 100 ppm. Controls received distilled water. This concentration was selected based on the result of a preliminary test.

4.3.2 *In vitro* Test Setup and Inhibition Measurement

The paper disc assay (De Beer and Sherwood 1945) was applied with some modifications, as follows. A plug of 6 mm diameter of each fungus from the growing margin of the culture grown on PDA was placed on (Becton, Dickinson And Company, Sparks, MD, USA) in a 9 cm Petri dish containing fresh PDA. A 13 mm disc of sterile Whatman paper was placed at 13 mm distance from the margin of fungal plug. One hundred micro liters of Lance at 100 ppm a.i. or a mixture of Headline, Bravo and Lance each at 100 ppm a.i. were poured on the paper

disc, whereas controls received 100 µL of distilled water. Plates were kept at room temperature in the dark. After 48 h, the diameter of fungal mycelium close to the fungal disc was measured and subtracted from the average diameter of the control. The degree of fungal growth inhibition was calculated as percent inhibition (compared with control). Increase in fungal mycelium diameter was presented as a negative value of percent inhibition.

4.3.3 Statistical Analysis

The growth inhibition data were subjected to ANOVA to assess the significance of difference in fungal sensitivity to fungicides, followed by a Tukey *post hoc* test to assess the difference between growth inhibition means using SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA) (1999).

4.4 Results

The three fungicide mixture of Bravo, Lance and Headline clearly inhibited the growth of all the fungi (Table 4.1), but the degree of growth inhibition was highly variable among different fungal taxa (Figure 4.1). The fungi most sensitive to the mixture of three fungicides were *G. graminis* and *Trichoderma* OTU 10-3; whereas, *F. redolens*, *Setophoma*, *F. acuminatum* and *Bionectria* sp. were the least-sensitive fungi (Figure 4.1).

Table 4.1 Significance of the difference in responses of 12 fungal isolates to fungicide treatments in *vitro* according to ANOVA.

Source	DF	F Value	P Value
Experiment mixture of three fungicides			
Sensitivity of fungal isolates	11	19.56	<0.0001
Experiment fungicide Lance			
Sensitivity of fungal isolates	11	3.32	0.0067

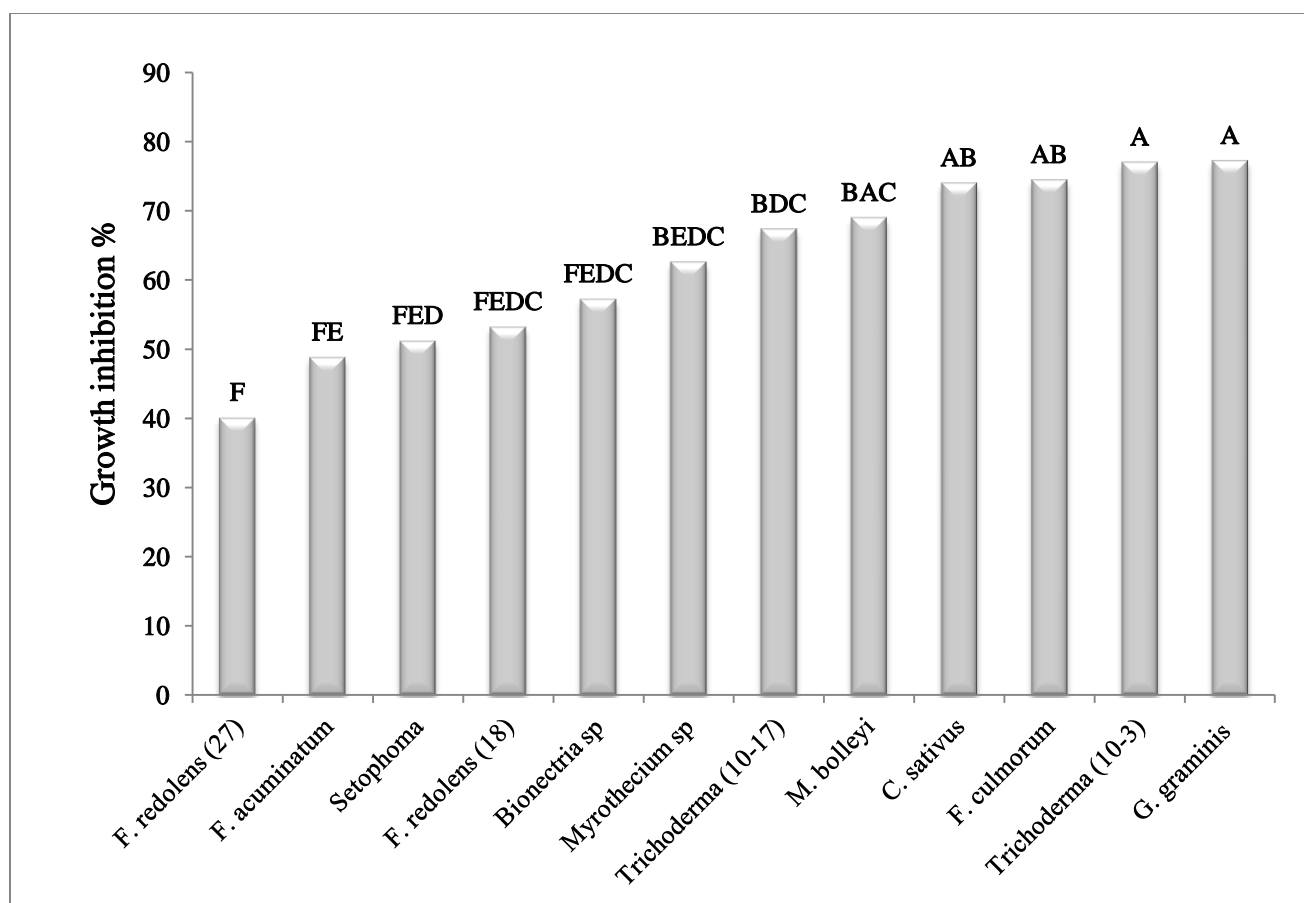


Figure 4.1 Growth suppression of different fungal species by 100 ppm a.i. liquid mix of Headline, Lance and Bravo after 48 h, bars with different letters are significantly different, $n = 3$.

Lance, which was the only fungicide detectable in rhizosphere soil (Yang et al. 2012b), also affected fungal growth (Table 4.1), but it had a different pattern of effect on fungal growth compared with the mixture of three fungicides (Figure 4.2). Variation was observed in the sensitivity of different fungi to fungicides. For instance, *Trichoderma* OTU 10-17 was most sensitive to Lance (Figure 4.2). The aggregate fungicide tolerance of the highly virulent pathogens (i.e. *F. culmorum*, *F. acuminatum*, *G. graminis* and *C. sativus*) and fungal antagonists (i.e. *Trichoderma* spp., *Bionectria* sp. and *Myrothecium* sp.) was similar for both combinations of fungicides (data not shown).

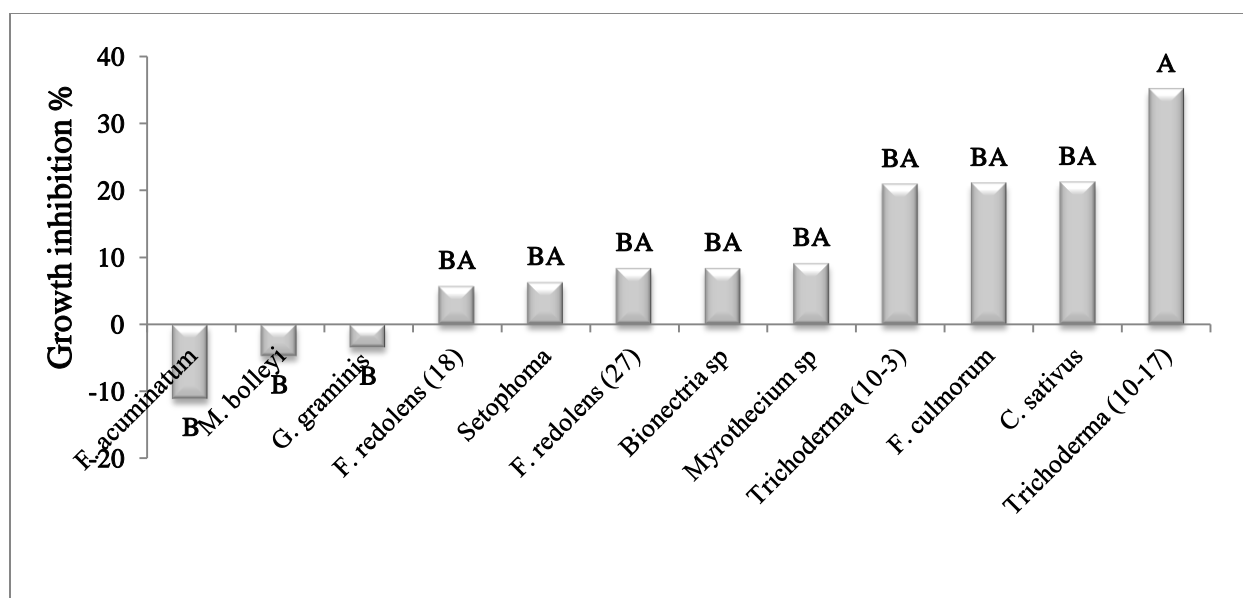


Figure 4.2 Growth suppression of different fungal species by 100 ppm a.i. of Lance after 48 h, bars with different letters are significantly different, $n = 3$.

4.5 Discussion

All the fungi were suppressed by fungicide treatment but the level of suppression differed among species. Two species of *Fusarium*, *F. redolens* and *F. acuminatum*, were among the species least-sensitive to the mixture of fungicides; whereas, *F. culmorum* was highly-suppressed by the same treatment. The pattern of growth inhibition was different when only the fungicide Lance was added to the medium. For example, *G. graminis*, was highly-suppressed by the mixture of three fungicides but tolerant to Lance. The *in vitro* effect of Lance is of special interest because this is the only fungicide detected in rhizosphere soil at the end of the chickpea growing season (Yang et al. 2012b), suggesting that Lance reaches the rhizosphere and directly influences the rhizosphere community. *Fusarium acuminatum* was resistant to both fungicide treatments (i.e. Lance alone and the mixture of three fungicides), which is in agreement with the findings of Elmer (1996), who reported the failure of several fungicides to inhibit hyphal growth in this species.

Some species responded differently to direct exposure to fungicides *in vitro* compared with their reaction to fungicide in the field. For instance, *F. culmorum* was very sensitive to fungicide *in vitro* but its abundance in the field was unaffected by fungicide use. However,

fungi antagonists and highly virulent pathogens responded similarly to exposure to fungicide both when the mixture of three fungicides or Lance alone were used. The fact that fungal antagonists and highly virulent pathogens of durum roots responded differently to the field application of fungicide on previous chickpea suggests that factors other than direct growth suppression may play a role in the field. One possible indirect impact may result from occupation of different niches by different functional groups which would define the vulnerability of each functional group to environmental disturbance, including fungicide contamination. For instance, it is known that some root and crown pathogens inhabit previous year stubble and attack new crops from there (Bockus and Shroyer 1998; Wearing and Burgess 1977). Occupying stubble may give pathogens an advantage to avoid high doses of fungicide residue in the soil; whereas, free living soil saprotrophs may be harmed directly and more seriously by fungicide (Robinson et al. 2005). Current results suggest that fungicide application may give advantages to specific fungi, including certain root and crown pathogens. Lance had minimal or no effect on the growth of some very important root pathogens such as *Gaeumannomyces graminis* and *Fusarium acuminatum*, and it clearly suppressed the growth of *Trichoderma* OTU 10-17, which may raise concerns about the effects of fungicides on soil borne pathogen built up. Lance may be more of a concern as it is a more persistent compound (Lagunas-Allué et al. 2010; Yang et al. 2012b).

4.6 Conclusion

The fungicide tolerance of fungal species varies and each combination of fungicide treatment affects fungal growth in a different way. However, highly virulent pathogens and fungal antagonists were similarly-impacted by fungicide exposure *in vitro*; whereas, they responded differently to foliar fungicide application in the field. This suggests that in the field the mechanism and the outcome of fungicide effect on fungi can vary between different functional groups.

4.7 Connection to the Next Study

To understand the role of the fungal communities in the poor performance of durum wheat after chickpea, in chapters three and four, I investigated the effects of fungicides on fungal communities in the field and the inhibition-effect of fungicides on important fungi *in vitro*.

Fungicide use is a necessary cropping practice in chickpea production. Although my results suggest that fungicide use in chickpea compromises the root health of the following durum wheat crops, fungicide use did not fully-explain the variation in productivity of durum wheat following chickpea. In the next study, I investigate the effect of previous crops of pea vs. chickpea as well as termination time of chickpea on the fungal community in the root of the following crop of durum wheat to reveal the likely changes in fungal communities that could explain the inferior performance of durum after chickpea compared to that after pea. As in chapter three, plate culture and pyrosequencing were used to examine the fungal communities associated with durum wheat root.

**5. ROTATION CROP AND CHICKPEA TERMINATION TIME IMPACT ON THE FUNGAL
COMMUNITY OF THE ROOT ENDOSPHERE OF A FOLLOWING DURUM WHEAT
CROP**

5.1 Abstract

Chickpea is commonly used in wheat-based cropping systems of southwestern Saskatchewan; however, the overall benefit of this legume to the following crop is smaller than that of pea. The influence of a crop plant on the health and productivity of cropping systems can be attributable to both the direct influence of the plants and to the influence of the agronomic practices used to grow the crop. A field study was used to disentangle the effects of pea and chickpea cropping, along with the effect of the termination time of chickpea, i.e. July and September, on the composition of the fungal community that becomes established in the endosphere of a crop of durum wheat following in rotation. The fungal communities in seminal and adventitious roots of durum wheat at heading were described by pyrosequencing of ITS amplicons. The culturable fungi hosted in healthy and diseased roots of the durum wheat at maturity were isolated and identified according to BLAST search in public databases. *Fusarium* were predominant in the roots of durum wheat, particularly following a crop of chickpea, while some other fungi such as *Phoma* were more frequently seen following pea. Basidiomycota was associated with using pea as rotation crop. The relative abundance of *Fusarium* was negatively correlated with durum wheat yield. The effect of previous crop and previous year chickpea termination time on the community of culturable fungi living in the endosphere of the following durum crop was insignificant. Antagonistic fungal taxa were more abundant after pea than after chickpea CDC Vanguard and CDC Luna. Chickpea CDC Vanguard increased the abundance of highly virulent pathogens in durum wheat endosphere compared to chickpea CDC Luna and pea. Improved root health attributes in pea rotation, i.e. less *Fusarium* and more fungal antagonists may explain the superior durum yields after pea.

5.2 Introduction

Pulses are frequently used in cereal-based cropping systems of Saskatchewan (Cruz et al. 2012; Gan et al. 2009). Chickpea, a drought-tolerant crop (Cruz et al. 2012; Pande et al. 2005) primarily cultivated in the Brown and Dark Brown soil zones in southern Saskatchewan (Gan et al. 2009; Vandenberg 1998), is more profitable than many other crop plants, including pea (Saskatchewan Ministry of Agriculture 2012). However, wheat often yields less after chickpea than after pea (Miller et al. 2002).

The crops used in rotation influence soil chemistry, physics and biology with impacts on the performance of the crops that follow (e.g. Katsvairo et al. 2002; Kirkegaard et al. 2004). Since crop plants differ in phytochemistry (Cruz et al. 2012), phenology and microbial compatibility (Andreote et al. 2010; Gan et al. 2007; Prévost and Bromfield 2003; Xu et al. 2009), and since they are grown using specific farming practices, their effects on the soil environment are different. For example, chickpea leaves less nitrogen to the subsequent crop and consumes more soil water due to its longer growing season and deeper roots (Miller et al. 2003). However, difference in water use and nitrogen fertilization used could not completely explain the lower productivity of wheat crops after chickpea than after pea (Miller et al. 2002). The soil biota is a key component of the ecosystem with impact on plant health and productivity that may be altered by rotation crops (Anderson and Parkin 2007; Kirkegaard et al. 2004). In fact, crop rotation is the primary means of soil-borne disease control in agro-ecosystems (Baker and Cook 1974; Cook 2007; Janvier et al. 2007). Some rotation crops are more efficient than others in suppressing pathogens or enhancing beneficial microorganisms (Fernandez 2007; Kirkegaard et al. 2004). The microbial legacy of a crop is determined by the characteristics of crop and farming practices associated with its production (Ahemad and Khan 2010; Prévost and Bromfield 2003; Yang et al. 2012b).

Pulses have become a popular alternative to summer fallow in diversified cereal-based cropping systems of Saskatchewan (Gan et al. 2009; Gan et al. 2003; Miller et al. 2003), but there are relatively few studies on their influences on the following crop (Miller et al. 2002). Furthermore, the majority of the published studies have focused on factors other than soil microbiology (e.g. Gan et al. 2003; Miller et al. 2002), and very few included the soil microbial component. Yang et al. (2012a) have studied the effects of pulse management practices on soil bacterial communities in Saskatchewan. They found that late pulse termination time favors the proliferation of Firmicutes in wheat roots, but the effect of pulse rotation and related farming practices on the diversity and functionality of the fungi establishing in the roots of following wheat is unknown.

Fungi are the predominant component of the soil microbiota (Ruzicka et al. 2000; Schnürer et al. 1985) and they are involved in several key functions in ecosystems (Anderson

and Parkin 2007). Saprophytes are essential in organic matter decomposition and nutrient cycling (Robinson et al. 2005). The primary causes of disease in crops are fungal pathogens (Reeleder 2003), which may cause tremendous yield losses (e.g. McMullen et al. 1997). In contrast, fungal endophytes and arbuscular mycorrhizal fungi can enhance plant nutrition and health and improve productivity (Jeffries and Rhodes 1987; Ownley et al. 2008).

I hypothesized that pea and chickpea plants, and/ or the different termination time of these crops, influence the composition of the fungal communities associated with the roots of a following durum wheat crop, with influence on productivity. Therefore, the goal of this study was to reveal the influence of previous crops and previous chickpea termination time on the fungal communities of wheat roots to find out if shifts in root fungal community could explain differences in subsequent wheat yield. Fungal communities of asymptomatic and symptomatic roots by plate culture method were analyzed separately to study treatment effects on root endophytes and pathogens in a precise way (Wilson 1995). In pyrosequencing, seminal and adventitious roots were analyzed independently to take into account the differences in time of emergence and the position in the soil of seminal and adventitious roots.

5.3 Materials and Methods

5.3.1 Experimental Site, Design and Farming Management

The experiment was conducted in 2008-2009 and repeated in 2009-2010 in a chickpea, pea – durum wheat rotation system at the Semiarid Agricultural Research Centre, in Swift Current, Saskatchewan (latitude 50° 18' N; Longitude 107° 41' W). The average precipitation from January until the end of August was 46.3 mm month⁻¹ in 2008, 28.38 mm month⁻¹ in 2009, and 60.88 mm month⁻¹ in 2010. There were five levels of previous crops randomized in blocks (1- chickpea CDC Vanguard terminated at maturity, 2- chickpea CDC Luna terminated at maturity 3- chickpea CDC Vanguard terminated one month earlier at the time of pea maturity, 4- chickpea CDC Luna terminated one month earlier at the time of pea maturity, and 5- pea CDC Handel terminated at maturity. Treatments were randomized in four complete blocks in 2 m x 8 m plots.

For durum wheat cultivar, seeding date, harvest date, seed treatments and fertilization information please see section 3.3.1. The chickpea plots growing the previous year had received five applications of fungicides (for detailed fungicide treatment information please see section 3.3.1).

The culturable fungal communities of healthy and diseased durum roots following pea CDC Handel, chickpea CDC Luna (two termination times) and CDC Vanguard (two termination times) were described using plate culture at wheat maturity. The communities of fungi in the seminal and adventitious roots of durum wheat were described at heading stage in relation to previous crops of pea, chickpea CDC Vanguard, and chickpea CDC Luna using pyrosequencing of ITS1.

5.3.2 Root Sampling and Processing

Please see section 3.3.2 for detailed methodology.

5.3.3 PCR and PCR Product Preparation for Pyrosequencing

Please see section 3.3.3 for detailed methodology.

5.3.4 Sequence Management, Clustering and Taxonomical Annotation

Please see section 3.3.4 for detailed methodology.

5.3.5 DNA Extraction, PCR and Fungal Identification

Please see section 3.3.5 for detailed methodology.

5.3.6 Construction of Functional Group

Please see section 3.3.6 for detailed methodology.

5.3.7 Statistical Analysis

The significance of previous crop treatment effect was tested using Permutation-based MANOVA (perMANOVA) on the data of plate culture and pyrosequencing as explained in section 3.3.7. To visualize the effects of year, root health and rotation treatment on the fungal communities and the nature of interaction between fungal taxa Non-metric multidimensional scaling (NMDS) was applied using PC-ORD (MjM Software, Gleneden Beach, OR, USA) (McCune and Mefford 2011). For explanation about the interpretation of NMDS plots please see section 3.3.7. The procedure of rare species exclusion and data relativization was the same as described in section 3.3.7. Statistical analysis and statistical software used to explore the effect of rotation treatments on the fungal functional groups was similar to the section 3.3.7. The relationship between durum wheat yield and different fungal functional or taxonomical groups was examined according to Spearman Correlation test in R 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria) (2011).

5.4 Results

5.4.1 Pyrosequencing

At heading, significant interactions between previous crop and year, and between root type and year were observed (Table 5.1). NMDS revealed communities of fungi associated with durum roots were segregated based on previous crop (Figures 5.1 and 5.2). However, the significant interaction of year and previous crop suggests the pattern differs in 2009 and 2010 (Figures 5.1 and 5.2). *Fusarium*, the predominant fungal genus, was closely associated with cultivation of chickpea, specifically CDC Vanguard in both years (Figures 5.1 and 5.2).

ANOVA revealed that the genus *Fusarium* was significantly more abundant in adventitious roots of durum wheat following chickpea (Figure 5.3, Table 5.2) than pea. Basidiomycota seemed to be more abundant in durum root after pea (Figure 5.4) and Glomeromycota (arbuscular mycorrhiza fungi) constituted less than one percent of durum root mycota (data not shown).

Table 5.1 Effect of different previous crops on the overall fungal community of durum root endosphere detected by pyrosequencing at heading stage, according to perMANOVA, $N= 48$.

Source	Df	F Value	P Value
Previous crop	1	3.3380	0.001
Root type	1	3.2386	0.001
Year	1	7.4769	0.001
Previous crop X Root type	1	1.7060	0.073
Previous crop X Year	1	2.2730	0.014
Root type X Year	1	4.0234	0.001
Previous crop X Root type X Year	1	1.4213	0.165

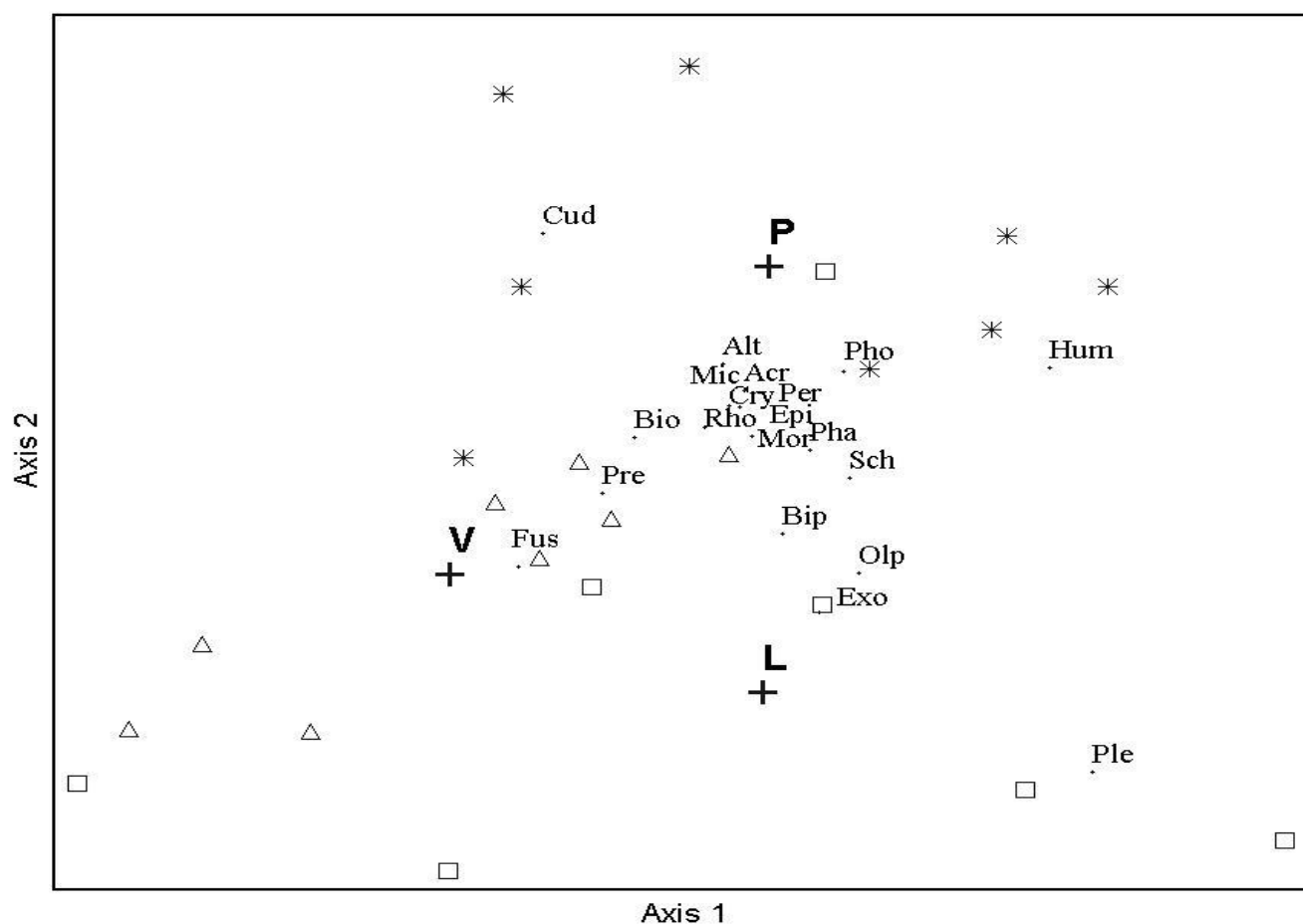


Figure 5.1 NMDS bi-plot of the fungal community associated with durum roots after different rotation crop at early heading stage in 2009 according to pyrosequencing. Stress = 14.17, $P = 0.02$, N (the total number of plots) = 24. L = chickpea CDC Luna, V = chickpea CDC Vanguard, P = pea CDC Handel. Triangles = CDC Vanguard, rectangles = CDC Luna, stars = Pea. Variance explained by axis 1 = 0.269 and by axis 2 = 0.573. Fus = *Fusarium*, Cry = *Cryptococcus*, Mic = *Microdochium*, Olp = *Olpidium*, Per = *Periconia*, Alt = *Alternaria*, Exo = *Exophiala*, Mor = *Mortierella*, Pha = *Phaeosphaeria*, Pre = *Preussia*, Pho = *Phoma*, Bio = *Bionectria*, Acr = *Acremonium*, Bip = *Bipolaris* (*Cochliobolus*), Rho = *Rhodotorula*, Ple = *Plectosphaerella*, Hum = *Humicola*, Epi = *Epicoccum*, Cud = *Cudoniella*, Sch = *Schizothecium*.

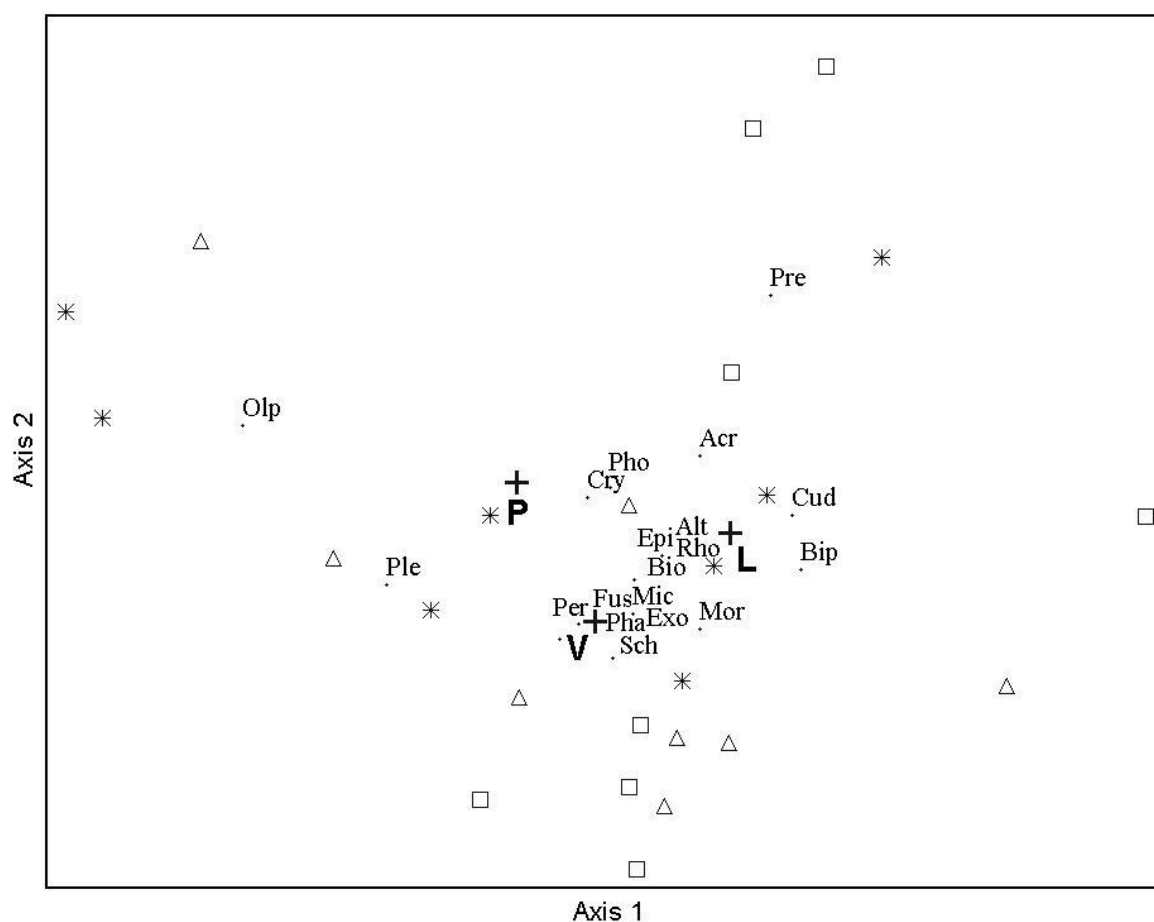


Figure 5.2 NMDS bi-plot of the fungal community associated with durum roots after different rotation crop in early heading stage in 2010 according to pyrosequencing. Stress = 16.55, $P = 0.02$, N (the total number of plots) = 24. L = chickpea CDC Luna, V = chickpea CDC Vanguard, P = pea CDC Handel. Triangles = CDC Vanguard, rectangles = CDC Luna, stars = Pea. Variance explained by axis 1 = 0.445 and by axis 2 = 0.380. Fus = *Fusarium*, Cry = *Cryptococcus*, Mic = *Microdochium*, OLP = *Olpidium*, Per = *Periconia*, Alt = *Alternaria*, Exo = *Exophiala*, Mor = *Mortierella*, Pha = *Phaeosphaeria*, Pre = *Preussia*, Pho = *Phoma*, Bio = *Bionectria*, Acr = *Acremonium*, BiP = *Bipolaris* (*Cochliobolus*), Rho = *Rhodotorula*, Ple = *Plectosphaerella*, Hum = *Humicola*, Epi = *Epicoccum*, Cud = *Cudoniella*, Sch = *Schizothecium*.

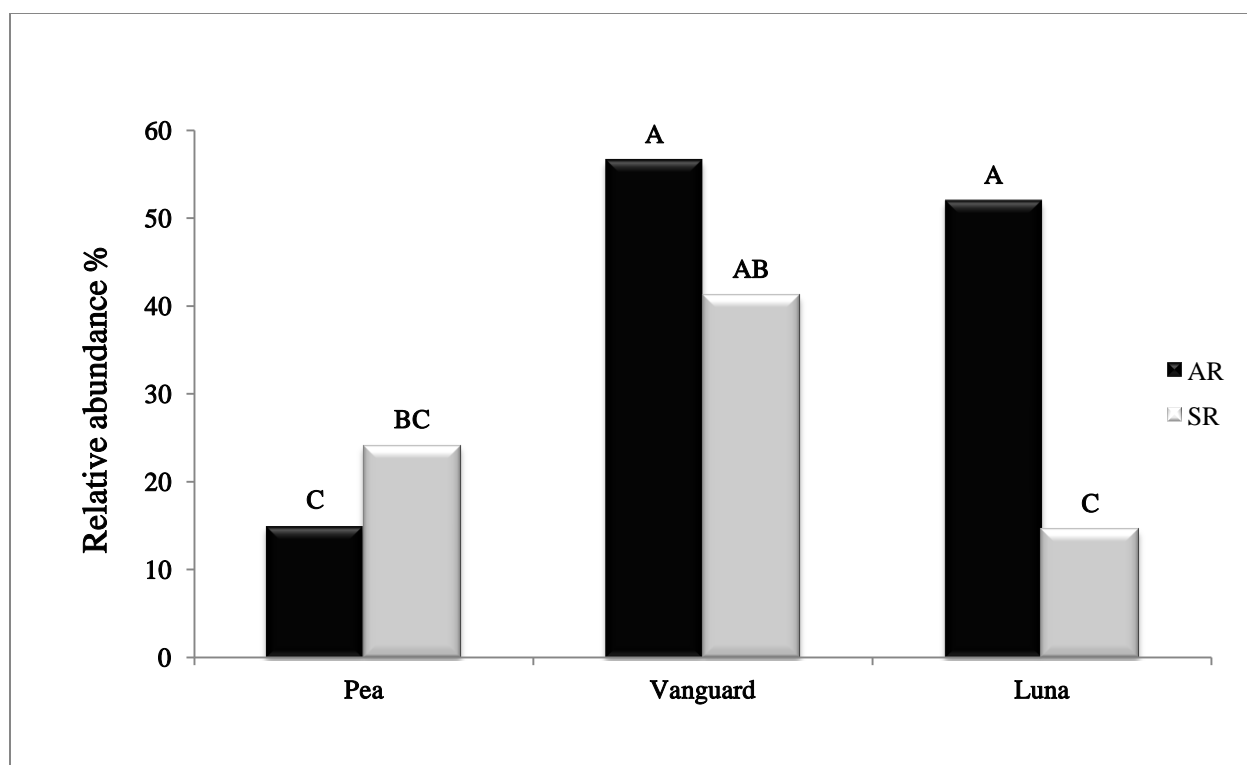


Figure 5.3 Effect of different previous crops and durum root type on the relative abundance of the genus *Fusarium* associated with the adventitious roots (AR) and seminal roots (SR) of durum wheat at heading stage as detected by pyrosequencing. Bars with different letters are significant at $P < 0.05$, $n = 8$.

Table 5.2 Significance of the effect of previous crop, root type, year and their interactions on relative abundance of *Fusarium* spp. in durum roots at heading, according to mixed model analysis of the pyrosequencing data, $n = 4$.

Effect	DF	F Value	P Value
Year	1	12.22	0.0015
Previous crop	2	9.12	0.0008
Root type	1	8.22	0.0075
Year X Previous crop	2	3.50	0.0430
Year X Root type	1	0.89	0.3524
Previous crop X Root type	2	6.07	0.0061
Year X Previous crop X Root type	2	0.47	0.6279

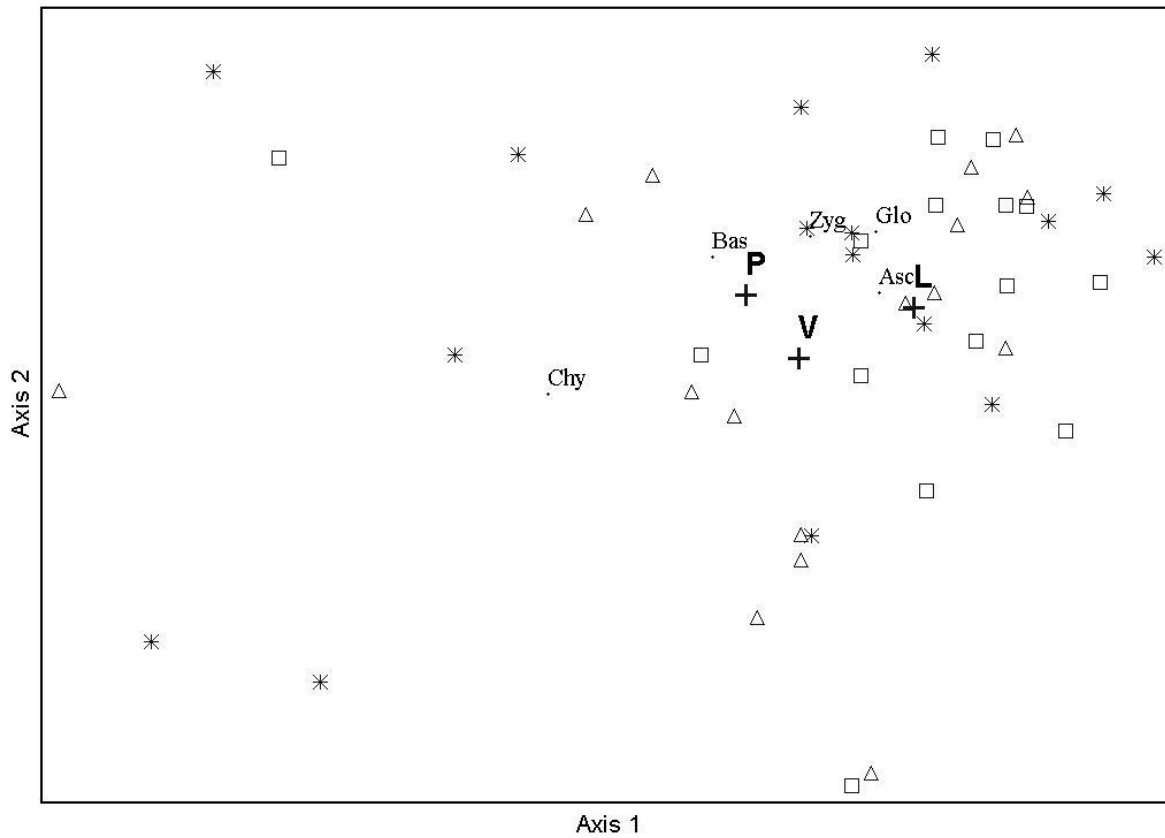


Figure 5.4 NMDS bi-plot of the fungal community in phylum level associated with durum roots in heading stage in 2010 according to pyrosequencing. Stress = 9.65, $P = 0.02$, N (the total number of plots) = 48. L = chickpea CDC Luna, V = chickpea CDC Vanguard, P = pea CDC Handel. Triangles = CDC Vanguard, rectangles = CDC Luna, stars = Pea. Variance explained by axis 1 = 0.766 and by axis 2 = 0.196. Asc = Ascomycota, Bas = Basidiomycota, Chy = Chytridiomycota, Glo = Glomeromycota, Zyg = Zygomycota.

A negative correlation was found between durum yield and the relative abundance of *Fusarium* in the root endosphere of wheat at heading (Figure 5.5).

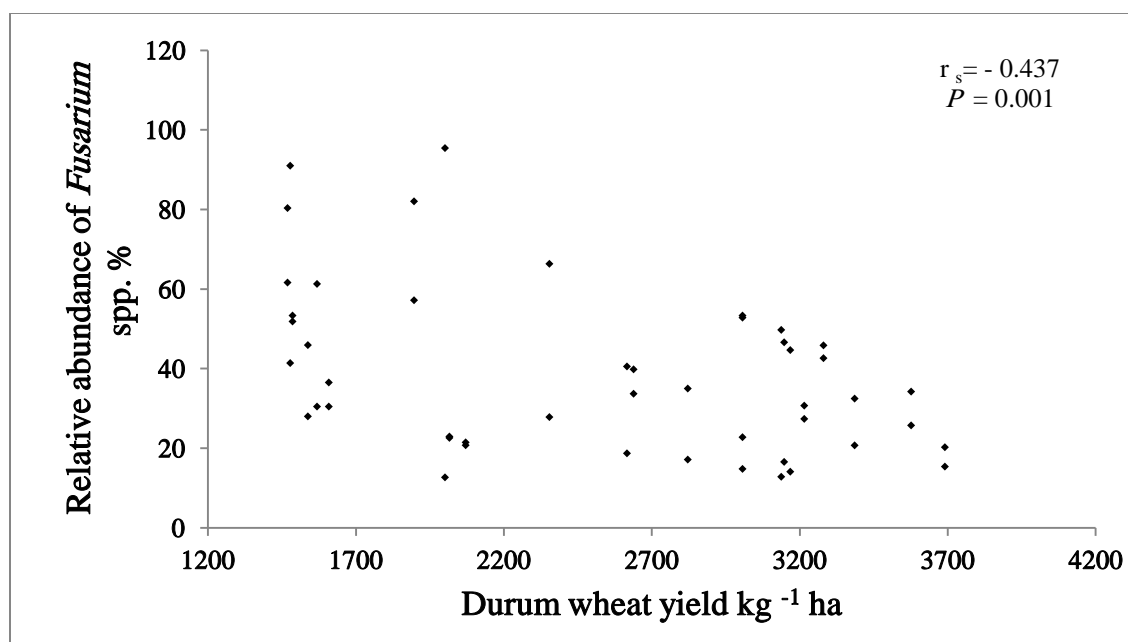


Figure 5.5 The correlation of *Fusarium* relative abundance and durum yield according to the Spearman correlation test, N (the total number of plots) = 48.

5.4.2 Plate Culture

The interaction of root health status and year on the community structure of root fungi was significant (Table 5.3). Dark septate endophytes (DSE) were dominant in healthy roots, but rare in symptomatic roots in 2009 and 2010 as revealed by NMDS (Figure 5.6- 5.7).

Table 5.3 The effect of previous crop, previous chickpea termination time, year and root health status on overall fungal community of durum root at maturity stage detected by plate culture in 2009 and 2010 as shown by perMANOVA, $N = 80$.

Source	Df	F Value	P Value
Previous crop treatment	4	0.8470	0.743
Year	1	25.7044	0.001
Root health status	1	17.5770	0.001
Previous crop treatment X Year	4	1.0601	0.404
Previous crop treatment X Root health status	4	1.1723	0.246
Root health status X Year	1	17.9312	0.001
Previous crop treatment X Root health status X Year	4	0.7482	0.864

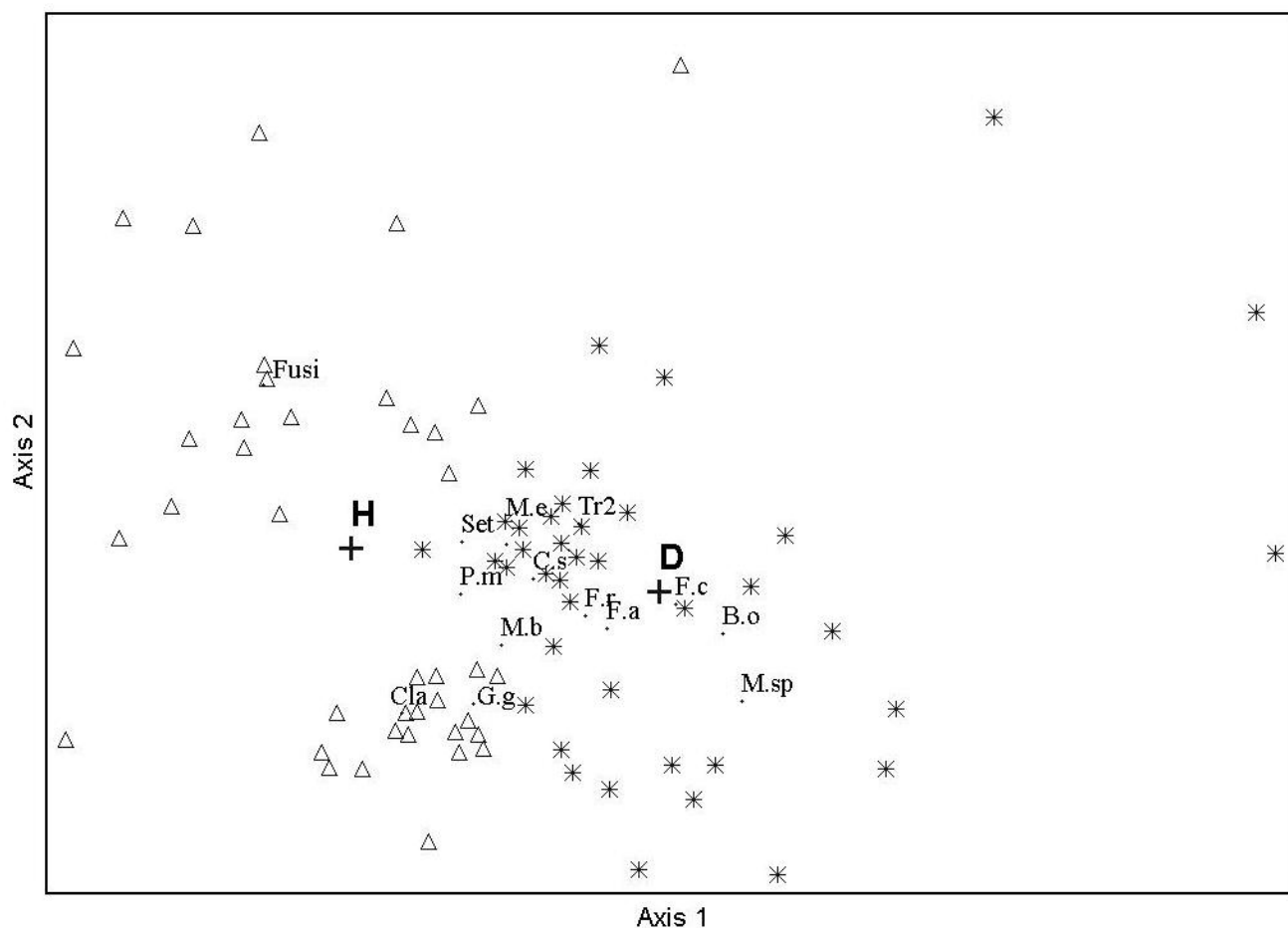


Figure 5.6 NMDS plot of the fungal community associated with healthy (triangles) and diseased (stars) roots of durum wheat at the maturity stage according to plate culture, H = healthy, D = Diseased, $P = 0.02$, N (the total number of plots) = 80, stress = 16.7. Variance explained by axis 1 = 0.375 and by axis 2 = 0.494. C.s = *Cochliobolus sativus*, F.r = *Fusarium redolens*, F.c = *Fusarium culmorum*, G.g = *Gaeumannomyces graminis*, P.m = *Periconia macrospinos*, F.s = *Fusarium solani*, Set = *Setophoma* sp. M.sp = *Myrothecium* sp, B.o = *Bionectria* sp, F.a = *Fusarium acuminatum*, Tr2 = *Trichoderma* (10-17), M. b = *Microdochium bolleyi*, Cla = *Cladosporium* sp., Fusi = *Fusidium* sp..

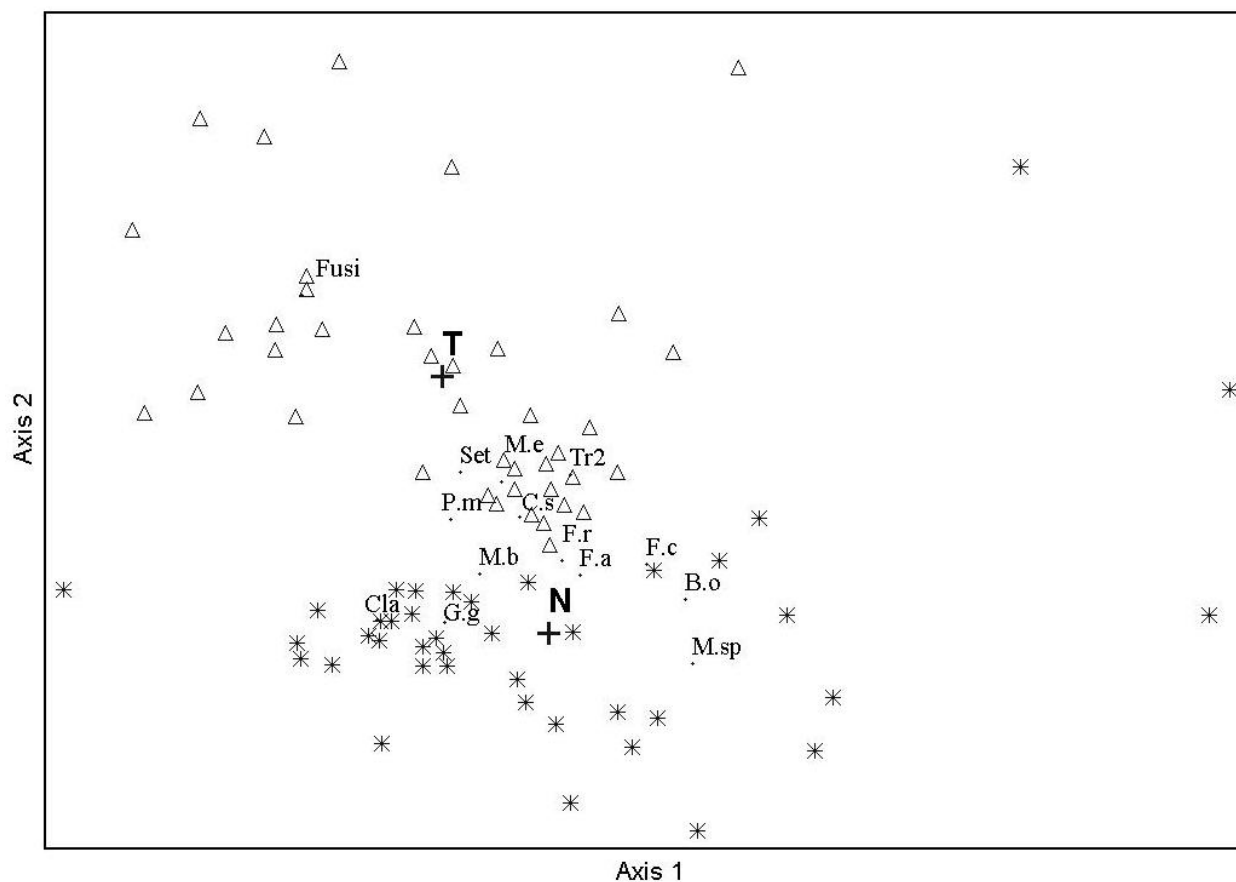


Figure 5.7 NMDS plot of the fungal community associated with the roots of durum wheat in 2009 (stars) and 2010 (triangles) at the maturity stage according to plate culture. N = 2009, T = 2010. $P = 0.02$, N (the total number of plots) = 80, stress = 16.7. Variance explained by axis 1 = 0.375 and by axis 2 = 0.494. C.s = *Cochliobolus sativus*, F.r = *Fusarium redolens*, F.c = *Fusarium culmorum*, G.g = *Gaeumannomyces graminis*, P.m = *Periconia macrospinoso*, F.s = *Fusarium solani*, Set = *Setophoma* sp. M.sp = *Myrothecium* sp, B.o = *Bionectria* sp, F.a = *Fusarium acuminatum*, Tr2 = *Trichoderma* OTU (10-17), M. b = *Microdochium bolleyi*, Cla = *Cladosporium* sp., Fusi = *Fusidium* sp..

At durum wheat maturity, the community of culturable endospheric fungi was not significantly influenced by previous crop treatments (Table 5.3). ANOVA revealed, however, that fungal functional groups responded differently to the previous crop. The fungal antagonists were more abundant after pea than chickpea (Table 5.4, Figure 5.8).

Table 5.4 Significance of the effect of rotation crop, year, and their interactions on fungal antagonists of symptomatic durum root at maturity, as shown by mixed model analysis of plate culture data.

Effect	DF	F Value	P Value
Year	1	0.14	0.7162
Previous crop	2	7.03	0.0095
Previous x Year	2	0.01	0.9855

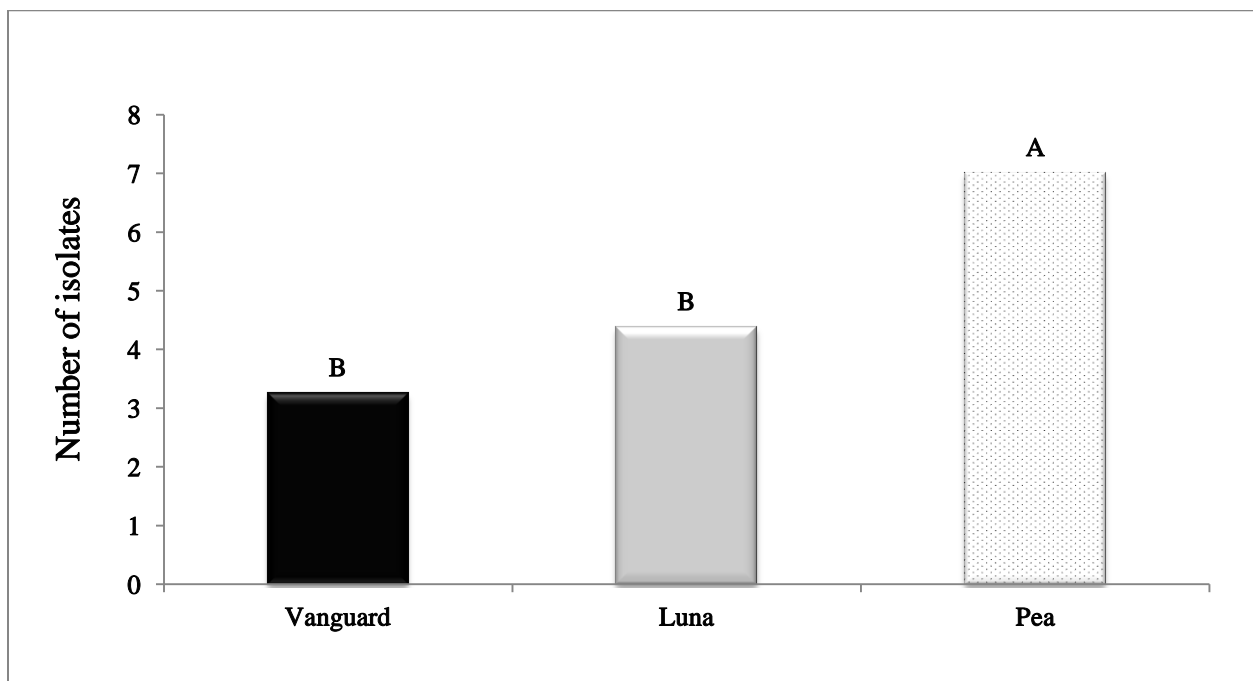


Figure 5.8 Effect of previous crop on the abundance of fungal antagonists in the root endosphere of durum wheat plants following in rotation. Bars with different letters are statistically significant according to LSD at $P < 0.05$, $n = 8$.

Durum roots after chickpea CDC Vanguard harbored more highly virulent pathogens (Table 5.5, Figure 5.9). Terminating chickpea at pea maturity (one month before normal chickpea harvest time) did not affect fungal community structure or functional groups associated with subsequent durum root at maturity (data not shown).

Table 5.5 Significance of the effect of previous crop, year, and their interactions on highly virulent pathogens of durum root at maturity, according to mixed model analysis of plate culture data.

Effect	DF	F Value	P Value
Year	1	0.07	0.7986
Previous crop	2	0.0252	0.0252
Previous crop x Year	2	0.6192	0.6192

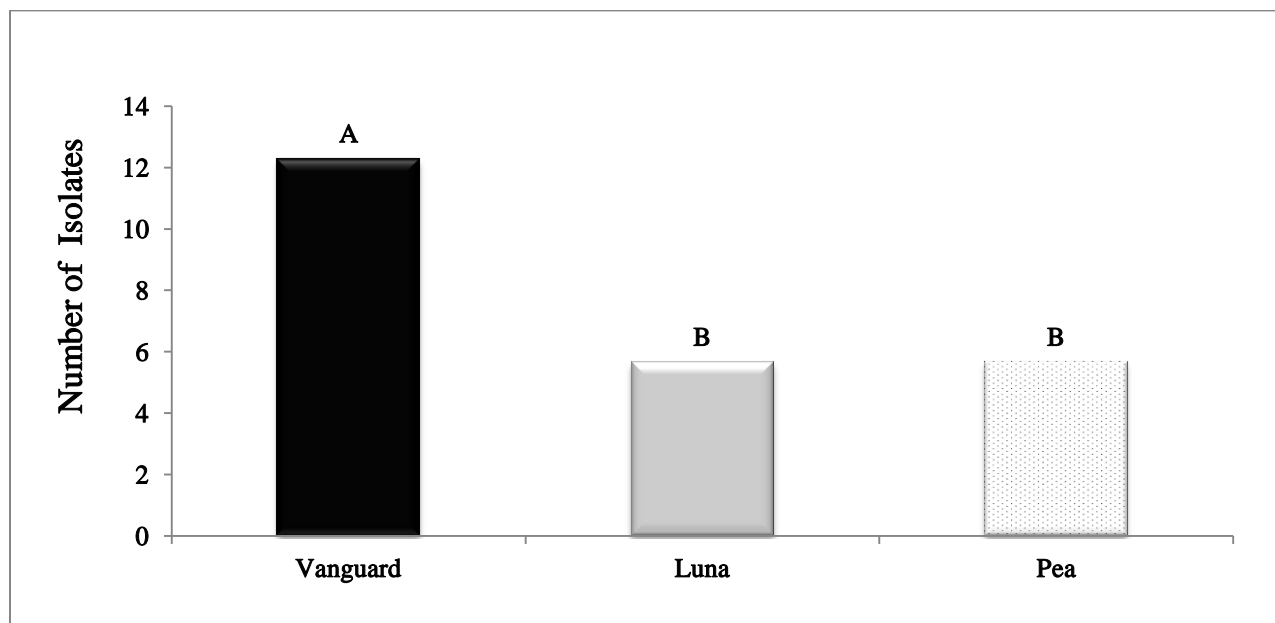


Figure 5.9 Effect of previous crop on the abundance of culturable highly virulent pathogens in the endosphere of durum wheat plants following in rotation. Bars with different letters are statistically significant at $P < 0.05$ according to LSD, $n = 8$.

There was a negative correlation between yield and the abundance of highly virulent pathogens in symptomatic durum wheat root (Figure 5.10).

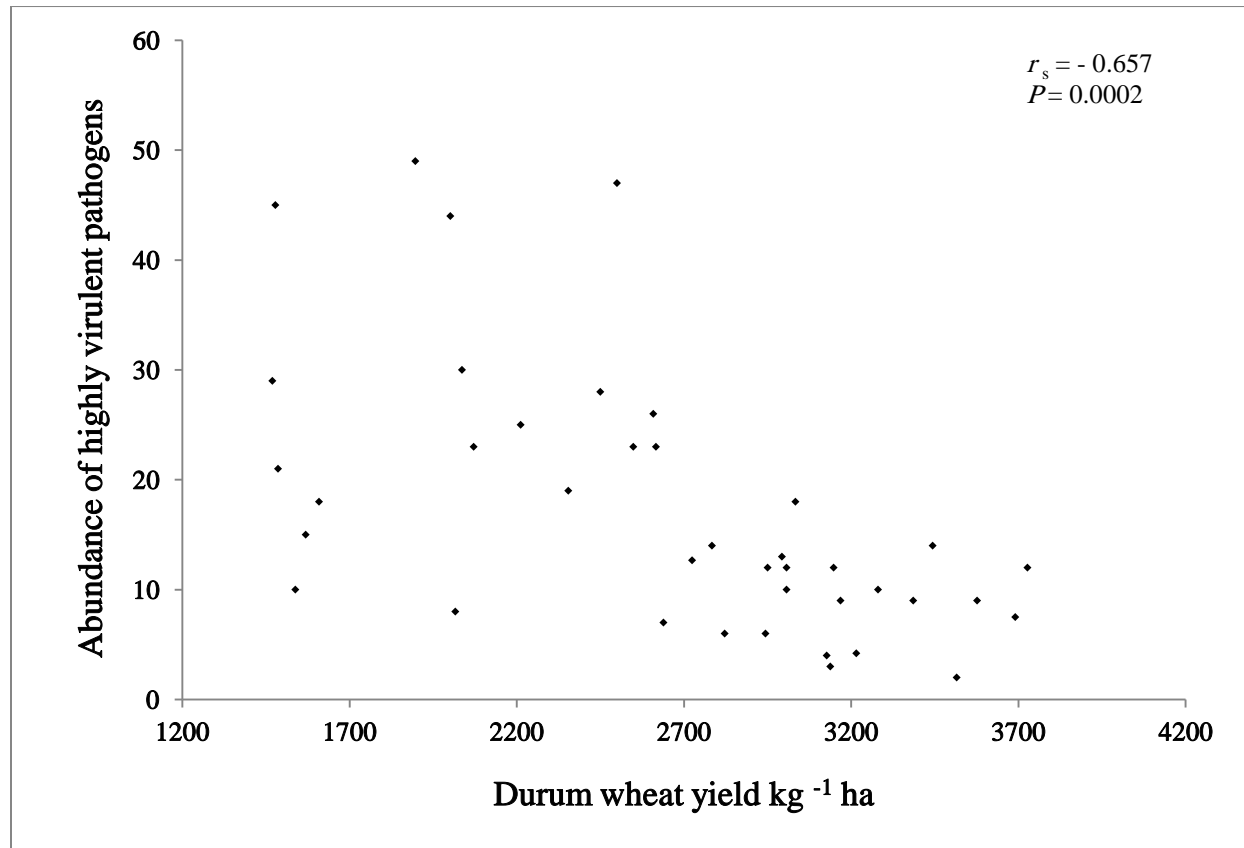


Figure 5.10 Negative correlation of durum yield with the abundance of highly virulent pathogens invading diseased roots of durum wheat as detected by plate culture, N (the total number of plots) = 40.

5.5 Discussion

Fusarium spp. were more abundant in the adventitious roots of wheat after chickpea compared with pea but there were no differences in the population of this genus in seminal roots in relation to using pea or chickpea in previous year according to pyrosequencing. Seminal roots of durum wheat after chickpea CDC Luna harboured fewer *Fusarium* compared to CDC Vanguard. *Fusarium* is a well known pathogenic species (Bockus and Shroyer 1998; Paulitz et al. 2002; Summerell et al. 2010; Summerell and Leslie 2011), therefore, higher populations of this

genus in durum roots following chickpea may cause yield reduction. Kirkegaard et al. (2004) have shown the incidence of crown rot caused by *F. pseudograminearum* was usually higher in wheat after chickpea than of the brassicas. They suggested lower degradation of cereal stubbles in chickpea plots as a reason for higher incidence of *F. pseudograminearum* nevertheless surface stubbles (mainly cereals) in the pre-seeding stage of my experiment in 2009 were not different in pea and chickpea (data not shown). However chickpea residues placed in the soil degrade slower than pea because pea residues have lower C:N ratios (Lafond et al. 2000). Furthermore late chickpea maturity means long-lasting canopy shading on the soil and lower soil temperature (Bockus and Shroyer 1998) which may slow down biological degradation. Incomplete degradation of chickpea residues means more *Fusarium* inocula for the succeeding crop (Fernandez and Fernandes 1990) with possible adverse effect on plant health. It is shown that *Fusarium* spp. in subterranean parts of pulses and spring wheat in Saskatchewan were similar which supports this hypothesis (Fernandez 2007; Fernandez and Jefferson 2004). Another explanation for high populations of *F. pseudograminearum* after chickpea suggested by Kirkegaard et al. (2004) is a lower population of antagonists after chickpea. While I observed such a pattern in plate culture experiments, the relative abundance of antagonistic taxa in pyrosequencing data was very low and there was no significant correlation between them and *Fusarium* spp.

Chickpea and pea associate preferentially with different microorganisms (Ahemad and Khan 2010; Prévost and Bromfield 2003) and produce different defense compounds against pathogens (Hadwiger 2008; Stevenson et al. 1997; Ye et al. 2002); thereby, they may promote different groups of fungi. This means the creation of a different microbial legacy of chickpea and pea to succeeding wheat.

Phoma was one of the taxa closely associated with using pea as previous crop which was generally in agreement with the findings of Persson et al. (1997) who showed this genus was predominant in pea roots in southern Scandinavia. Although *Phoma* is a known pathogen of some crops, such as pea (Persson et al. 1997), some isolates of *Phoma* are shown to promote plant growth in wheat (Shivanna et al. 1996) and Koike et al. (2001) have shown improved resistance to *Fusarium* wilt and bacterial leaf spot in cucumber (*Cucumis sativus* L.) as a result of inoculation with *Phoma*. This may provide an explanation for the better performance of durum

wheat after pea. However, the taxonomy and functionality of *Phoma* is very complicated (Montel et al. 1991; Persson et al. 1997; Shivanna et al. 1996). Therefore, confident judgment on the identity and functionality of *Phoma* in this experiment was not possible, particularly since the short ITS sequences obtained from pyrosequencing were limiting. In addition to *Phoma*, some other fungal taxa such as *Phaeosphaeria*, *Acremonium*, *Humicola*, and *Cudoniella* were associated with using pea as previous crop. Higher durum yield after pea may suggest these fungi have beneficial effects on the host such as improved mineral nutrition and/or resistance to biological and environmental stressors. More research in this regard is warranted.

Basidiomycota is closely-associated with using pea as previous crop. This taxon plays a central role in decomposition (Frankland et al. 1982) especially in degradation of complex compounds such as lignin (Robinson et al. 2005; Thormann 2006) which makes up 30-60% of organic matter in soil (Garrett 1951). The unique capability of Basidiomycota for lignin degradation (Robinson et al. 2005), along with higher soil temperature in pea plots after harvest (Bockus and Shroyer 1998), may result in a higher decomposition rate and more nutrients for subsequent wheat which in turn may improve the yield. Pugh (1980) suggested that more attention should be paid to the Basidiomycota due to their significant role in decomposition and long term availability of nutrients. In agreement with his view, I call for more attention to diversity and functionality of Basidiomycota in Canadian prairies.

According to plate culture data, pea rotation resulted in a more abundant community of fungal antagonists in mature durum root while chickpea CDC Vanguard enhanced the population of highly virulent pathogens. Lower stubble degradation rates in chickpea plots or different microbial preferences of pea and chickpea as explained before may be the reason for differences in populations of highly virulent pathogens and antagonists observed here. Regardless of the mechanism, both findings are in agreement with my initial hypothesis and could explain the poor performance of wheat after chickpea especially because of the negative correlation of durum yield with highly virulent pathogens. Kirkegaard et al. (2004) have shown higher incidence of crown rot in wheat after chickpea compared to brassicas. The increase in disease incidence was usually associated with lower population of fungal antagonists (*Trichoderma* spp.) which is similar to current results (Kirkegaard et al. 2004).

In this experiment, terminating chickpea at pea maturity time (one month before normal chickpea harvest time) did not have a significant effect on fungal community and functional groups associated with subsequent durum root at maturity according to plate culture (data not shown).

Members of the genera *Fusidium*, *Cladosporium*, *Lachnum*, *Setophoma* and *Ophiosphaerella* were more abundant in healthy roots which may suggest their beneficial effect on plant health through preventing pathogenic invasion or improving tolerance to abiotic stressors. Six species of fungal antagonists (potential biocontrol agents) were isolated in the course of this research: four species of *Trichoderma*, one species of *Bionectria*, one species of *Nectria* and one species of *Myrothecium*. The mycoparasitic interaction of most of these fungi on very well-known root pathogens isolated from the same field was observed in co-culture. There was also a negative correlation (although non-significant) between the abundance of this functional group and highly virulent pathogens (*C. sativus*, *G. graminis*, *F. acuminatum* and *F. culmorum*), suggesting a possible relevance of their effect in field condition. More research on inoculation effect of these fungi on plant health and productivity is warranted.

Current study suggests improved root health attributes in durum root after pea; however, other factors such as soil moisture and nutrients can also play role in better productivity of durum wheat following pea (Miller et al. 2002). Therefore, it is very likely that a combination of favorable conditions such as higher moisture and nutrients along with plant health result in a significantly higher durum yield after pea and not a single factor alone. More research is warranted to determine to what extent each factor is involved in the variation in durum yield after pea and chickpea.

Although the results of the current studies support the initial hypotheses and may provide complementary explanation for poor performance of durum wheat after chickpea, some limitations exist in the experiments. The plots of pea in the current experiment did not receive fungicide while chickpea plots received five fungicide treatments. While this is consistent with the common practice done by industry in southwestern Saskatchewan, fungicide application on chickpea may have confounded the effect of chickpea rotation. Also, short sequences provided by 454 pyrosequencing pose another major limitation in taxonomic identification of fungi at the

species level (i.e. is not sufficient), that effectively reduces resolution to that of genera-level identifications.

In summary, it seems part of the variation in durum wheat productivity after chickpea and pea in Saskatchewan's cereal-based agricultural systems could be explained by inferior root health attributes after chickpea, namely an increase in the population of root pathogens such as *Fusarium* in conjunction with lower numbers of fungal antagonists.

5.6 Conclusion

The previous crop affects the fungal communities associated with the roots of subsequent durum wheat. Increased root health (i.e. higher population of fungal antagonists and lower population of highly virulent pathogens) was observed in pea rotation which could explain higher durum productivity. Durum yield was negatively related to the population of pathogenic fungi. Chickpea genotype affects the population of soil pathogens and therefore is an important factor in shaping root health of the following crop. Previous crops affect the structure of fungal communities at the phyla level which may influence essential functionality of the ecosystem.

5.7 Connection to the Next Study

Fusarium redolens was very abundant in the roots of durum wheat, especially in the year 2009. Surprisingly, this fungus has not been reported to occur in Saskatchewan before. In the next study, I examined the abundance of this fungus on different crops in southwestern Saskatchewan. I also compared its pathogenicity with that of *C. sativus* and *F. graminearum* on various crops to provide a more comprehensive view of its economical importance.

6. FIRST REPORT OF *FUSARIUM REDOLENS* FROM SASKATCHEWAN AND ITS COMPARATIVE PATHOGENICITY

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Author contributions:

The experiment was designed by Ahmad Esmaeili Taheri with help from Vladimir Vujanovic. Phylogenetic tree and molecular identification of *Fusarium redolens* was performed by Vladimir Vujanovic. Chantal Hamel has helped and advised on the statistical analysis. The first draft of the paper was prepared by Ahmad Esmaeili Taheri, after which the other authors critically revised the manuscript. Paper was submitted by Vladimir Vujanovic.

6.1 Abstract

Fusarium redolens Wollenw. [syn: *F. oxysporum* var. *redolens* (Wr.) Gordon] is a pathogenic fungus on a wide range of plant species. *Fusarium redolens* was frequently isolated from necrotic and discolored root and crown of chickpea, pea, lentil, and durum wheat in Saskatchewan. The fungus was identified using distance analysis of the translation elongation factor-1 alpha sequences. Comparative pathogenicity test showed that *F. redolens*, like *Fusarium graminearum* Schwabe and *Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur, caused lesions and/or discolorations on the root, crown and shoot of durum wheat, pea and chickpea, although it had different host preference. While *F. redolens* was most virulent on pea, the other two fungi were more virulent on durum wheat. It appears that *F. redolens* is common in the Prairie Ecozone of Saskatchewan and it may cause yield reduction in pea, chickpea and durum crops. This warrants a closer look at the biology and ecology of *F. redolens* in the Canadian Prairies.

6.2 Introduction

Fusarium redolens is reported as a root and crown pathogen on a wide spectrum of plants (Gerlach and Nirenberg 1982). In Europe and the United States, this fungus was found associated with lentil (*Lens culinaris* Medik.) vascular wilt (Riccioni et al. 2008), with asparagus (*Asparagus officinalis* L.) (Baayen et al. 2000) and with soybean (*Glycine max* L.) root rot (Bienapfl et al. 2010). In Canada, *F. redolens* (reported as a variety of *Fusarium oxysporum*) was isolated from oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) seeds (Gordon 1952), and as one of the most frequent fungi isolated from agricultural soils in Manitoba (Gordon 1954). Also, this fungal pathogen is a member of the root rot complex, isolated from necrotic roots of lentil and pea (Sumar et al. 1982) in Alberta. A study of the effect of different rotation practices on the diversity of fungi associated with the roots of subsequent crops in southwest Saskatchewan recovered *F. redolens* from symptomatic roots and/or crown of pulses and durum wheat. Consequently, I decided to assess the presence of *F. redolens* on the different plant organs during specific phenophases. The symptoms on field-grown pulses include brown to black discoloration and/or lesions on crown, main and lateral

roots whereas in cereals adventitious roots were the main target of fungus where it caused light brown discolorations and lesions.

The virulence of *F. redolens* on pea, chickpea and durum wheat was compared with *F. graminearum* and *C. sativus* in phytotron studies.

6.2 Materials and Methods

6.2.1 Sampling and Fungal Isolation

Fusarium redolens was isolated from symptomatic roots and crown of chickpea (*Cicer arietinum* L.), pea (*Pisum sativum* L.), lentil, and durum wheat (*Triticum turgidum* var *durum* Desf.). The roots and crowns of the 30 to 50 plants dug up at each of the 14 samplings site/times (Table 6.1) were stored at 4°C for a maximum period of 48 h before processing. After thorough washing in tap water, 1- 3 cm segments of root or crown with visible lesions were surface sterilized in 96% ethanol (30 s), 20% commercial Javex (6% w:v sodium hypochlorite; 90 s), and rinsed in sterile distilled water four times, before excising and plating 8-mm segments on a solid medium containing 5 g L⁻¹ Potato Dextrose Agar (Becton, Dickinson And Company, Sparks, MD, USA), and 12 g L⁻¹ Bacto-Agar (Difco Laboratories Inc, Detroit, MI, USA) amended with 10 mL L⁻¹ 0.6% chlortetracycline, and 1.5 mL L⁻¹ 0.3% streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) (Burgess et al. 1988; Fernandez and Jefferson 2004; Vujanovic et al. 2002). Plates were incubated in the dark at 23°C, and hyphal tips of emerging fungi were sub-cultured on PDA amended with 1.5 mL L⁻¹ 0.3% streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA). Resulting isolates were subjected to sequential tip culture procedure to insure that the isolates were pure.

Table 6.1 Crops, sampling dates, crop growth stage, and relative abundance of *F. redolens* as percent of all culturable fungi obtained in all the plants, from the 14 sampling sites considered in this study (samples are taken from Swift Current, SK).

Site no	Plant (cultivar)	Previous Crop	Sampling Date	Plant growth stage	Frequency of <i>F. redolens</i> in %
Cereals					
1	Durum (AC Avonlea)	Pea	Aug 09	Ripening	31.5
2	Durum (AC Avonlea)	Chickpea	Aug 09	Ripening	18.8
3	Durum (AC Avonlea)	Pea	Jun 10	Booting	10.5
4	Durum (AC Avonlea)	Chickpea	Jun 10	Booting	8.8
5	Durum (AC Avonlea)	Pea	Aug10	Dough	14.2
6	Durum (AC Avonlea)	Chickpea	Aug 10	Dough	4.0
7	Durum (Eurostar)	Fallow	Aug 10	Dough	1.8
8	Durum (AC Avonlea)	Pea	Aug 10	Ripening	9.9
9	Durum (AC Avonlea)	Chickpea	Aug 10	Ripening	12.1
Pulses					
10	Pea (CDC Handel)	Spring Wheat	Jul 10	Mid-flower	27.8
11	Chickpea (CDC Vanguard)	Spring Wheat	Jul 10	Mid-flower	33.3
12	Pea (CDC Handel)	Spring Wheat	Aug10	Pea maturity	18.8
13	Chickpea (CDC Vanguard)	Durum Wheat	Aug10	Pea maturity	51.2
14	Lentil (CDC Richlea)	Spring Wheat	Sep 10	Maturity	33.3

6.2.2 DNA Extraction, PCR and Fungal Identification

Based on morphological characteristics about 200 isolates of *F. redolens* were grouped into two Operational Taxonomic Groups (OTU 18 and OTU 27). Five isolates from each group were subjected to DNA extraction using Qiagen DNeasy Plant DNA Extraction Kit (Qiagen Inc., Valencia, CA, USA). The internal transcribed spacer regions (ITS rDNA) and the translation elongation factor-1 alpha (EF-1 alpha) genes were amplified using primer pairs ITS1F (Gardes and Bruns 1993) /ITS4 and EF1/EF2 (O'Donnell et al. 1998; White et al. 1990) using a 96 well PCR thermocycler machine (Eppendorf AG, Hamburg, Germany). PCR conditions were: 94°C for 3 min, 30 cycles of 94°C for 30 s, 57°C for 35 s, 72°C for 20 s, and a final extension of 72°C for 10 min for the ITS region, and for EF1/EF2: 94°C for 3 min, followed by 30 cycles of 94°C for 30s, 57°C for 30 s, 72°C for 1 min and a final extension of 72°C for 10 min with some modifications from Mavragani (2008) and Corredor (2011). Sequences were edited using software Staden (Staden et al. 2000) and BLASTed against NCBI (<http://blast.ncbi.nlm.nih.gov/>) using default settings. *Fusarium* sequences were also BLASTed

against FUSARIUM ID database (Geiser et al. 2004) (<http://board.fusariumdb.org/board2.php?a=dv&nu=42&id=2>) to identify the closest (higher than 99% identity) sequences. The identity of two isolates of *F. redolens* was revealed through phylogenetic analyses including the most similar *F. redolens* translation elongation factor alfa-1 sequences in NCBI, as well as sequences of *Fusarium hostae* Geiser & Juba and *Fusarium oxysporum* Schltdl. : Fr. as outgroup. A distance tree was produced with PAUP (phylogenetic analysis using parsimony) 4.0b10 program (Swofford 2003) (Figure 6.1), using a neighbor-joining approach, and validated using bootstrap analyses with 1,000 repetitions. The elongation factor alfa-1 sequences of *F. redolens* OTU18 and OTU 2G7, and the ITS sequences of *C. sativus* were deposited in GenBank with the accession numbers JF27261, JF272612 and JF272613, respectively. The above-mentioned fungi are deposited in Saskatchewan Microbial Collection and Database (SMCD) with strain numbers: SMCD 2401, SMCD 2402, and SMCD 2403, respectively.

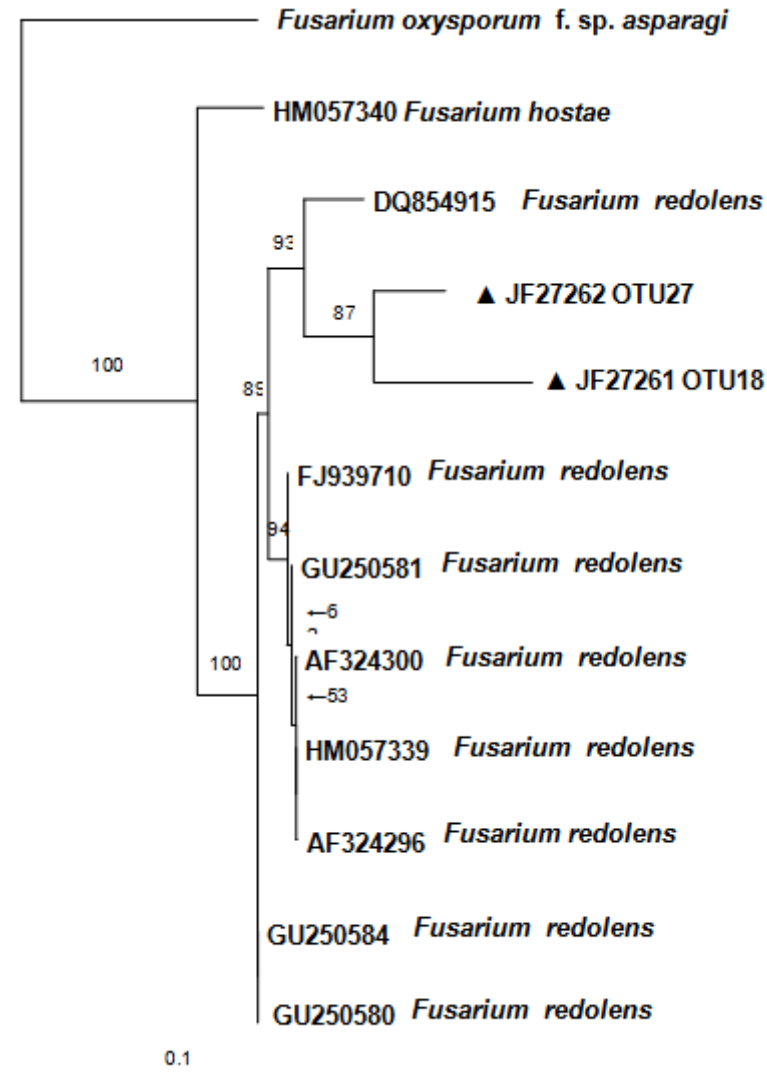


Figure 6.1 Phylogenetic tree constructed from the elongation factor 1 α (EF-1 alpha). Data set showing the clustering of *F. redolens*, OTU 27 and OTU 18 (▲) sequences. *F. hostae* and *F. oxysporum* f. sp. *asparagi* sequences retrieved from GenBank. Bootstrap values from 1,000 replicates are indicated above the nodes.

6.2.3 Pathogenicity Test

I compared the pathogenicity of the two above-mentioned *F. redolens* strains, OTU 18 (SMCD 2401) and OTU 27 (SMCD 2402), both isolated from durum wheat, to that of *C. sativus* SMCD 2403 and of *F. graminearum* (3-ADON) SMCD 2243 on seedlings of pea (CDC Handel), chickpea (CDC Vanguard) and durum (AC Avonlea). A factorial experiment with six replicates

was conducted under controlled conditions using a protocol modified from Molina (1979). Twenty five mL of a peat: vermiculite (1:2) mix and 9 mL of PD broth (Becton, Dickinson And Company, Sparks, MD, USA) (1 g L^{-1}) were added to 52-mL test tubes (14 cm x 2.5 cm) and autoclaved (Figure 6.2). A 7-mm disc from the margin of a fungal culture was placed 1 cm below the surface of the growth mix, in each inoculated tube, and control tubes received a sterile PDA disc. Tubes were pre-incubated at 24 °C for 6 days before aseptically planting one healthy looking seedling per tube. Seedlings were produced from surface sterilized (96% ethanol 30 s, 33% Javex 2 min, four rinses in sterile distilled water) seeds, aseptically plated on water agar (7 g L^{-1}), and incubated at room temperature. Tubes were capped with parafilm (Fisher Scientific, Hampton, NH) and their rooting zone covered with aluminum foil to protect roots and fungi from light. Tubes were kept under a 16 h/8 h day/night photoperiod with a light intensity of $215 \mu\text{mol m}^{-2} \text{ s}^{-1}$, a 22 °C/18 °C day/night temperature regime, and 50% relative humidity (Convion, Winnipeg, MB, CA). When appropriate, the parafilm was cut under aseptic conditions to free the plant shoot, and the tubes were re-capped with a new piece of parafilm, surrounding the shoot. After 25 days, plant disease severity was rated using a scoring method modified from Xue et al. (2007) (Table 6.2).

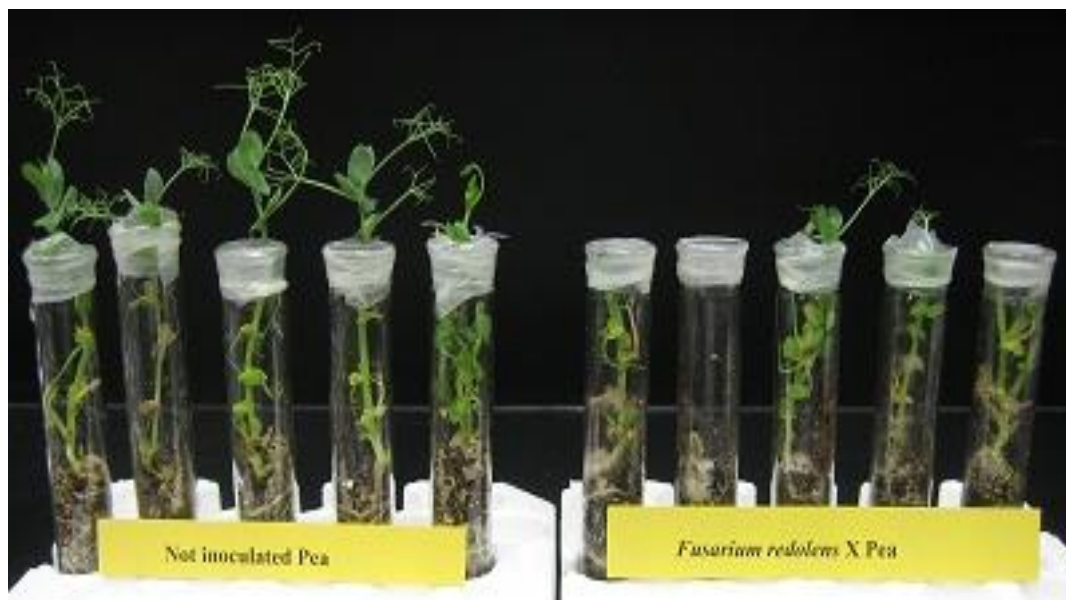


Figure 6.2 The effect of inoculation with *F. redolens* (SMCD 2402) on pea. Non-inoculated (left) and inoculated (right).

Table 6.2 Disease scoring index used in the pathogenicity test.

Health status of plants	
No visible symptom	0
Limited visible lesions or discoloration on root, chlorosis in leaves with normal plant size	1
Extended lesions or discoloration on root and/or shoot with < 50% reduction in plant size	2
Severe lesion or discoloration on root and/or shoot with > 50% reduction in plant size	3
Dead plant	4

6.2.4 Statistical Analysis

A General Linear Model (GLM) was fit to the disease-severity rating data after a Square root transformation for improving normality in SAS/STAT version 9.1 (SAS Institute Inc., Cary, NC, USA) (1999). Ls-Means were separated using Macro PDGLM 800 (Saxton 1998) based on Least Significant Difference (LSD).

6.3 Results and Discussion

In this study, *F. redolens* was frequently isolated from several fields in the Prairie Ecozone of Saskatchewan (Gauthier and Wiken 2003) in 2009 and 2010 (Table 6.1). The fungus was relatively abundant in roots, especially in pulses where it accounted for up to 51.2 % of all culturable fungi in chickpea roots (Table 6.1). Two distinctive operational taxonomic groups of *F. redolens* were recognized. OTU 27 formed a woolly white mycelium and included about 80% of *F. redolens* isolates. OTU 18 had cream-white mycelium and changed the color of the medium to a pink color. Elongation factor alfa-1 sequences of these two OTUs were BLASTed against NCBI and Fusarium ID databases (Geiser et al. 2004) and in both databases the first hits were always *F. redolens* (more than 99 identity and 99.5% query coverage). Also, in phylogenetic analysis the sequences obtained from above-mentioned isolates were clustered with the sequences of *F. redolens* (Figure 6.1)

In the pathogenicity test isolates of *F. redolens* caused brown to black discoloration and/or lesions on the roots and crowns of chickpea and pea, and in some cases the whole seedling was invaded and dead. On durum wheat, *F. redolens* isolates caused brown discoloration in most parts of the root and the overall root development seemed suppressed. In

one case, leaves were completely invaded and a light brown lesion developed throughout the entire leaf. The roots of chickpea inoculated with *C. sativus* had black discoloration and lesions, and seeds and leaves were invaded in some cases. On pea, *C. sativus* caused brown to black discoloration and lesions on the roots. *Cochliobolus sativus* produced intense black and brown lesions on durum roots, and in most cases, the leaves of the plant were completely invaded. *Fusarium graminearum* produced black to brown lesions on pea and chickpea roots, crown and seeds, and very-intense lesions and discoloration on the roots and crowns of durum wheat, with almost all aerial parts being dead. Controls were symptomless. The fungi emerging from surface-sterilized (96% ethanol 20 s, and 10% Javex 1 s, sterile distilled water rinse) lesioned tissues plated on PDA, were identified as the fungi introduced in the tubes, fulfilling Koch's postulates. The symptoms developed on plants in *in vitro* tests correspond with those observed on field-grown hosts.

The different fungal species caused different levels of disease on the different host plants, as shown by a plant x fungus interaction ($P < 0.001$) found upon GLM analysis of the disease-rating data (non-inoculated controls with disease severity scores of zero for all three hosts were excluded). *Fusarium redolens* was not known as a pathogen of chickpea, but the virulence of this fungus on chickpea was medium, and similar to that of *C. sativus* (Figure 6.3), according to LSD. *Cochliobolus sativus* was previously isolated from field-grown chickpea in Saskatchewan (Holzgang and Pearse 2000), but there is no report of this fungus on pea (Farr and Rossman 2010). *F. redolens* isolate SMCD 2402 was most virulent on pea and SMCD 2402 was as virulent as *F. graminearum* (3-ADON) on this plant, in agreement with previous studies reporting pea as a major host for *F. redolens* (Gerlach and Nirenberg 1982). Durum wheat was less susceptible to *F. redolens* SMCD 2402 than to *F. graminearum* and *C. sativus*, two potent cereal root pathogens (Dyer et al. 2009; Gonzalez and Trevathan 2000). The virulence of the *F. redolens* strains on durum wheat ranged from high (SMCD 2401) to low (SMCD 2402) indicating potential strain preferences to the plant species or genotype (Figure 6.3).

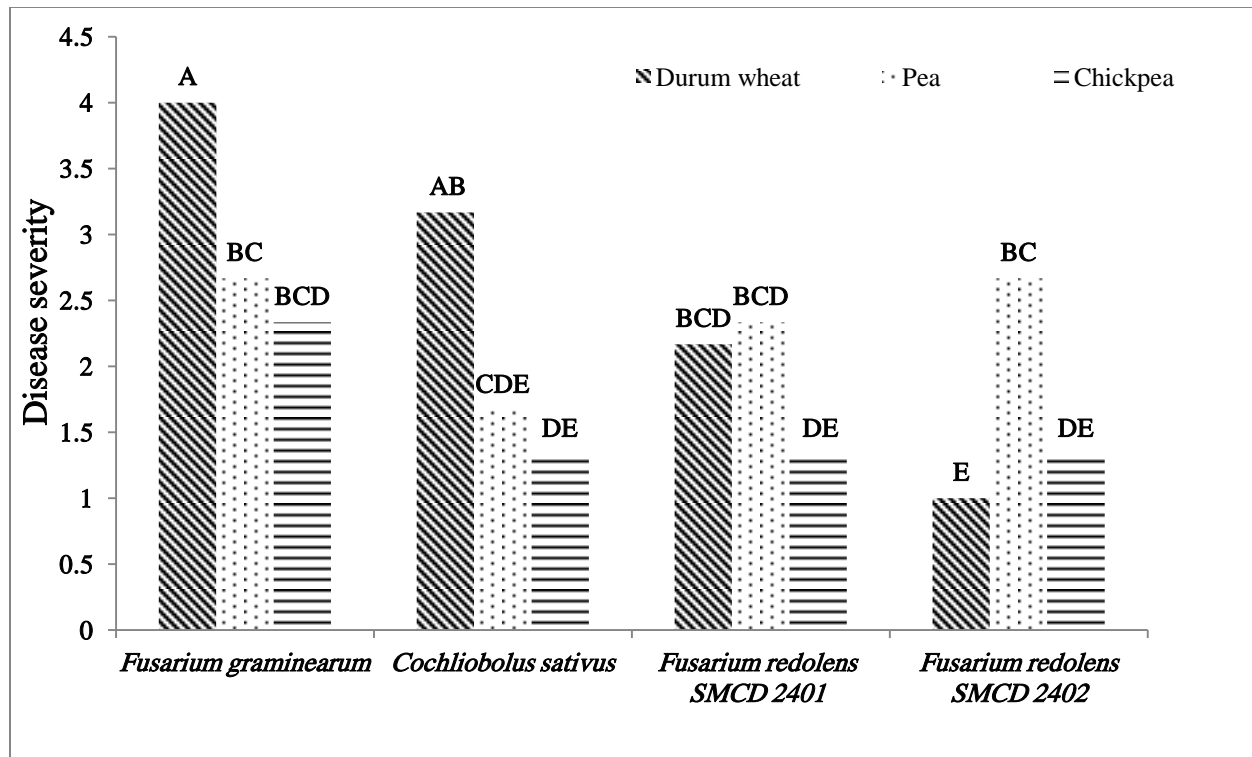


Figure 6.3 Comparative pathogenicity of *F. redolens*, *F. graminearum* and *C. sativus* on chickpea, durum wheat and pea. Disease-severity rating is based on the 0-4 scale described in Table 2. Least square means with the same letter are not significantly different at $P < 0.05$, $n = 6$.

High virulence of *F. redolens* strains isolated from durum wheat on pea suggests a wide host range as a feature of this fungal species, which contrasts with a previous report (Baayen et al. 2000). This may suggest that a comprehensive study to document host specificity and virulence of *F. redolens* among Saskatchewan crops is needed in order to understand its economical significance and provide appropriate control measures.

Fusarium redolens is reported as one of the most frequent *Fusarium* taxa in the soils of the prairies provinces of Manitoba to the east of Saskatchewan (Gordon 1954). It also has been reported from Alberta (Sumar et al. 1982) and British Columbia (Punja et al. 2007) to the west of Saskatchewan. This may indicate that *F. redolens* is a frequent component of the soil mycobiota of the Great Plains of Canada, and supports my conclusion to the likely wide-occurrence of this fungus in Saskatchewan. To my best knowledge, this is the first report of *F. redolens* from Saskatchewan. The identification of *Fusarium* species based on colony and fungus morphology

is difficult since *F. redolens* is very similar to *F. oxysporum* (Leslie and Summerell 2006); this may explain why this species is absent from the reports on the fungi associated with root diseases in pulse and cereal crops in Saskatchewan. Increasing use of pulses in wheat-based rotation system throughout the Canadian prairies over the last two decades may have increased the incidence of *F. redolens* in Saskatchewan. Around four million hectares of land are planted with durum wheat, pea, chickpea and lentil crops in Saskatchewan each year (www.statcan.gc.ca). The observation of *F. redolens* for the first time in Saskatchewan on all these crops indicates the need to clarify the biology and ecology of this fungus in order to provide adequate information regarding its economical significance and adoption of appropriate control measures.

6.4 Conclusion

Fusarium redolens, a fungal pathogen reported here for the first time from Saskatchewan, is fairly-abundant in the southwestern part of the province. This species especially is abundant on pulses. Virulence of this species on different crops varies. *Fusarium redolens* possesses an intermediate virulence on durum wheat whereas its virulence on pea is as high as *F. graminearum* and *C. sativus*.

6.5 Connection to the Next Study

Predominance of *F. redolens*, a new species to Saskatchewan, on the roots of durum wheat was unexpected. *Cochliobolus sativus* is known as the main cause of root rot disease of cereals in the province. Evaluation of cereal root rot and isolation of responsible pathogen(s) using classic methods is often done by the examination of the subcrown internode which is a subterranean stem. My hypothesis is that the discrepancy in my results with that of others is due to the use of a different plant organ in isolating the responsible agent (root vs. subcrown internode). In the next study, I examined fungal communities associated with different subterranean organs of wheat across Saskatchewan to test this hypothesis.

**7. NICHE SPECIFICITY OF THE FUNGAL COMMUNITIES INVADING THE
SUBTERRANEAN ORGANS OF WHEAT CALLS FOR CAUTION IN ROOT AND
CROWN DISEASES DIAGNOSIS**

7.1 Abstract

Root and crown diseases of wheat are caused by a number of fungi that target adventitious roots, seminal roots, crowns, and subcrown internodes. The definition of diseases affecting root and crown is inconsistent and different subterranean organs are used to assess severity and isolate responsible agent(s). The differences in anatomy and position in the soil of the subcrown internode, crown, seminal roots and adventitious roots may lead to differences in the fungal communities associated with them. Using plate culture, the distribution of fungi within the subterranean organs of field-grown wheat across Saskatchewan was examined to clarify the interactions involved in root and crown diseases in this important wheat-growing region. Plate culture revealed that the fungal communities hosted by wheat subterranean organs were different, except for seminal and adventitious roots, which hosted similar communities. *Cochliobolus sativus* was predominant in symptomatic subcrown internodes but rare in seminal and adventitious roots where *Fusarium* spp. were predominant. The fungal community of the subcrown internode, which is often used for diagnosis of root rot, was not representative of the community hosted by roots. The sole examination of wheat subcrown internode may lead to the underestimation of the involvement of *Fusarium* spp. in root rot disease.

7.2 Introduction

The root and crown diseases of wheat, which involve roots, crown, and subcrown internode (Smiley et al. 2005), are a major limitation to yield (Cook 2007; Ledingham et al. 1973; Smiley et al. 2005; Wildermuth et al. 1992). Two of the most important diseases affecting the root and crown, common root rot and crown rot, are inconsistently defined. Many researchers considered *Cochliobolus sativus* and *Fusarium* spp. to be the causative agents of common root rot (e.g. Chen et al. 1996; Hill et al. 1983; Sallans 1965; Tobias et al. 2009), but others define common root rot as an infection caused by *C. sativus* and use the names “Crown rot”, “Fusarium root rot”, or “Dryland root rot” when *Fusarium* spp. are the main disease-causing agents (e.g. Fedel Moen and Harris 1987; Smiley et al. 2005; Wiese et al. 2000).

Different subterranean organs are used to quantify disease severity and to isolate the responsible pathogens. Many researchers have used the subcrown internode and the crown to quantify the diseases and isolate their causative agents (Fernandez and Jefferson 2004;

Fernandez et al. 2011; Tobias et al. 2009; Wildermuth and McNamara 1991; Wildermuth et al. 1997); whereas, others examined all plant organs, including roots (Amir et al. 1991; Chen et al. 1996; Diehl et al. 1982; Gonzalez and Trevathan 2000; Hajieghrari 2009).

The subterranean organs of cereals occupy different positions in the soil profile (Figure 7.1), and clearly differ in anatomy and physiology, which is critical from a fungal ecology point of view. Among wheat subterranean organs, roots are specialized in water and nutrient absorption, which is an essential function for plant growth. Damage to roots due to pathogen invasion could compromise plant water and mineral nutrient absorption, which may translate into significant yield losses. Despite the important function of roots, it is surprising that the majority of studies on root and crown diseases ignored them in the evaluation of disease severity and diagnosis (e.g. Dyer et al. 2009; Fernandez and Jefferson 2004; Fernandez et al. 2011; Tobias et al. 2009; Wildermuth and McNamara 1991; Wildermuth et al. 1997). Fedel Moen and Harris (1987) have shown heterogeneous distribution of fungal pathogens among the subterranean parts of cereals in Australia. Tinline et al. (1994) have reported that *C. sativus* was predominant in crown and subcrown internode, but rare in roots. I hypothesized that the identity of wheat subterranean organs is a factor determining the structure of associated fungal communities. Therefore, focusing on certain organs (e.g. subcrown internode) and the exclusion of roots in the evaluation of root and crown diseases could be misleading.

The fungal communities inhabiting symptomatic seminal roots, adventitious roots, crowns, and subcrown internodes of wheat plants grown throughout Saskatchewan were analyzed using plate culture. The preferential occupation of adventitious and seminal roots by *Fusarium* spp. and the dominance of *C. sativus* in the subcrown internode were revealed by plate culture combined with PCR. The composition of the pathogenic fungi invading seminal and adventitious roots, crown and subcrown internodes of wheat are different, thus understanding diseases associated with subterranean parts of wheat requires the examination of all subterranean organs together.

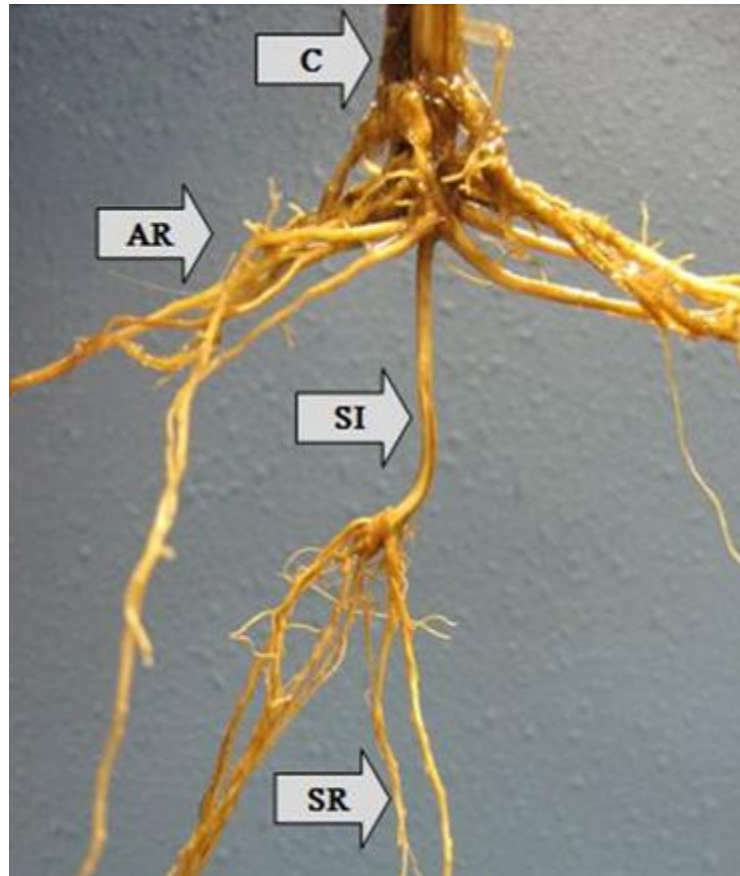


Figure 7.1 Architecture of wheat subterranean organs, C = Crown, AR = Adventitious roots, SI = Subcrown internode, and SR = Seminal roots.

7.3 Materials and Methods

7.3.1 Sampling, Plant Material Preparation and Fungal Isolation

Seminal roots, adventitious roots, subcrown internodes, and crowns (Figure 7.1) of different varieties of durum and spring wheat were collected from 19 different fields in Saskatchewan in 2009 and 2010 (Table 7.1). Between 30 and 40 plants were dug out from each location with a shovel; shoots were detached and roots were transported to the laboratory. Pieces of adventitious and seminal roots, crown, and subcrown internode with visible lesions were selected and then surface-sterilized by sequential soaking in ethanol (30 s), commercial Javex, and sterile distilled water (Burgess et al. 1988; Fernandez and Jefferson 2004; Vujanovic et al. 2002). The remaining material was dried at 60°C for 72 h and stored at room temperature.

According to Fernandez and Jefferson (2004) and Burgess et al. (1988) with some modifications, twenty fresh 8-mm segments of each adventitious and seminal roots, and five 8-mm segments of each of crowns and subcrown internodes were plated on solid medium made with 5 g L⁻¹ PDA (Becton, Dickinson And Company, Sparks, MD, USA) and 12 g L⁻¹ Bacto-Agar (Difco Laboratories Inc, Detroit, MI, USA). 10 mL L⁻¹ 0.6% chlortetracycline, and 1.5 mL L⁻¹ 0.3% streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) were added to the medium (Burgess et al. 1988; Fernandez and Jefferson 2004; Vujanovic et al. 2002). The Petri plates, each containing five segments, were incubated in the dark at room temperature. The emerging fungi were identified, sub-cultured on PDA (1.5 mL L⁻¹ of 0.3% w/v streptomycin sulfate, Sigma-Aldrich, St. Louis, MO, USA) (Vujanovic et al. 2002), and the number of isolates per 20 segments (abundance) was recorded.

Table 7.1 Variety, cropping history, geographic location, soil type and growth stage of the 19 wheat crops sampled in 2009 and 2010.

Year	Wheat variety	Previous crop	Municipality	Chernozem great group	Growth stage
2009	Avonlea	Chickpea or pea	Swift Current	Brown	Heading
2009	Avonlea	Chickpea or pea	Swift Current	Brown	Dough stage
2010	Avonlea	Chickpea or pea	Swift Current	Brown	Heading
2010	Avonlea	Pea	Swift Current	Brown	Dough stage
2010	Eurostar	Fallow	Swift Current	Brown	Dough stage
2010	AC Lillian	Fallow	Swift Current	Brown	Dough stage
2010	Avonlea	Chickpea or pea	Swift Current	Brown	Maturity
2010	AC Cadillac	Fallow	Moose Creek	Dark Brown	Heading
2010	AC Lillian	Alfalfa	Kingsley	Black	Heading
2010	Red Fife Heritage	Red clover	Vonda	Black	Heading
2010	AC Cadillac	Fallow	Duck Lake	Black	Heading
2010	AC Lillian	Mustard	Last Mountain Valley	Dark Brown	Heading
2010	Unknown	Unknown	Newcombe	Brown	Heading
2010	Unknown	Unknown	Newcombe	Brown	Heading
2010	AC Lillian	Sweet clover	Heart's Hill	Dark Brown	Heading
2010	AC Cadillac	Pea	Enniskillen	Dark Brown	Heading
2010	AC Elsa	Lentils	Snipe Lake	Brown	Heading
2010	Unknown	Unknown	Grant	Black	Heading
2010	AC Cadillac	Pea	Vanscoy	Dark Brown	Heading

7.3.2 Identification of Fungal Isolates

About 1 g of fresh fungal mycelium was taken from each morphotype and placed in 1.5 mL microcentrifuge tubes and left to dry at room temperature under sterile conditions for 48 h. Three sterile tungsten beads were added to each tube and the material was ground for 2 min in a Retsch® MM301 bead miller (Haan, Germany) at a stroke frequency of 20 s⁻¹. DNA was extracted from the ground samples using Qiagen DNeasy Plant Kits (Qiagen Inc., Valencia, CA, USA) according to manufacturer's protocol.

Different sets of primers were used (Table 7.2) in PCR reactions of 25 µL including 18 µL Platinum PCR Supermix from Invitrogen (Carlsbad, CA, USA), 3 µL distilled water, 1 µL of each primer and 2 µL of fungal DNA extract in a 96 well PCR thermocycler (Eppendorf AG, Hamburg, Germany). For PCR conditions please see section 3.3.5. PCR products were analyzed on agarose gel and successful amplifications were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol prior to Sanger sequencing at the NRC Plant Biotechnology Institute (PBI) in Saskatoon. Sequences were edited using Staden (Staden et al. 2000) and BLASTed in NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) at default settings. Fungal identifications were supported by sequence identity greater than 97% and morphological examination. Fungal strains were deposited in the Saskatchewan Microbial Collection and Database with collection numbers: SMCD 2401-2403, 2423-2427, and 2486. DNA sequences were deposited in the NCBI database with the following accession numbers: *Fusarium redolens* JF272611 and JF272612 (EF 1-alpha), *Fusarium culmorum* JQ658338 (EF 1-alpha), *Fusarium acuminatum* JQ658337 (EF 1-alpha), *Cochliobolus sativus* JF272613 (ITS rDNA), *Periconia macrospinosus* JQ658341 (ITS rDNA), *Microdochium bolleyi* JQ658340 (ITS rDNA), *Gaeumannomyces graminis* JQ658339 (ITS rDNA), *Setophoma* sp. JQ658342 (ITS rDNA).

Table 7.2 Primers used for fungal identification in this study.

Primer	Sequence	Reference
ITS-1F	CTTGGTCATTTAGAGGAAGTAA	(Gardes and Bruns 1993)
ITS2	GCTGCGTTCTTCATCGATGC	(White et al. 1990)
ITS-4	TCCTCCGCTTATTGATATGC	(White et al. 1990)
EF1	ATGGGTAAGGA(A/. G)GACAAGAC-	(O'Donnell et al. 1998)
EF2	GGAAGTACCAGTGATCATGTT	(O'Donnell et al. 1998)

7.3.3 Statistical Analysis

The relative abundance of each fungal species recovered in different plant organs was calculated as the number of isolates over the total number of fungal isolations in this organ, to allow the comparison of fungal abundance in the different organs. Plate culture data were relativized by row total using PC-ORD (MjM Software, Gleneden Beach, OR, USA) (McCune and Mefford 2011) to calculate relative abundance of each taxon in each organ.

Rare species (less than <3% of the total number of isolates) were excluded from the analysis of the plate culture data (McCune et al. 2002). Multi-response permutation procedure (MRPP) was used to test the significance of the effect of plant organ on the culturable fungal community (McCune et al. 2002) using PC-ORD (MjM Software, Gleneden Beach, OR, USA) (McCune and Mefford 2011). General Linear Model (GLM) was applied using SAS/STAT software, version 9.1 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) (1999) to test the effect of plant organ on fungal abundance. The significance of difference between means based on least significance difference (LCD) was assessed using Macro PDMIX 800 (Saxton 1998).

7.4 Results

The different subterranean organs of wheat hosted different fungal communities, except for adventitious and seminal roots, which hosted similar communities (Table 7.3). The distribution of the fungal species among plant organs was clearly uneven (Figure 7.2). In the subcrown internode, *C. sativus* was by far the most abundant fungus. *Fusarium* spp. were predominant in seminal and adventitious roots and *C. sativus* was rare especially in seminal roots. In crowns, *C. sativus* and *Fusarium* spp. were prevalent.

Table 7.3 MRPP comparison of the fungal communities associated with different subterranean organs of wheat.

Compared	Test statistics	P Value
Adventitious roots vs. Crown	-10.36633147	0.00000094
Adventitious roots vs. Subcrown internode	-9.71451953	0.00000602
Adventitious roots vs. Seminal roots	-1.33303181	0.10094725
Crown vs. Subcrown internode	-2.44330078	0.03129651
Crown vs. Seminal roots	-15.26492278	0.00000000
Subcrown internode vs. Seminal roots	-15.21548319	0.00000001

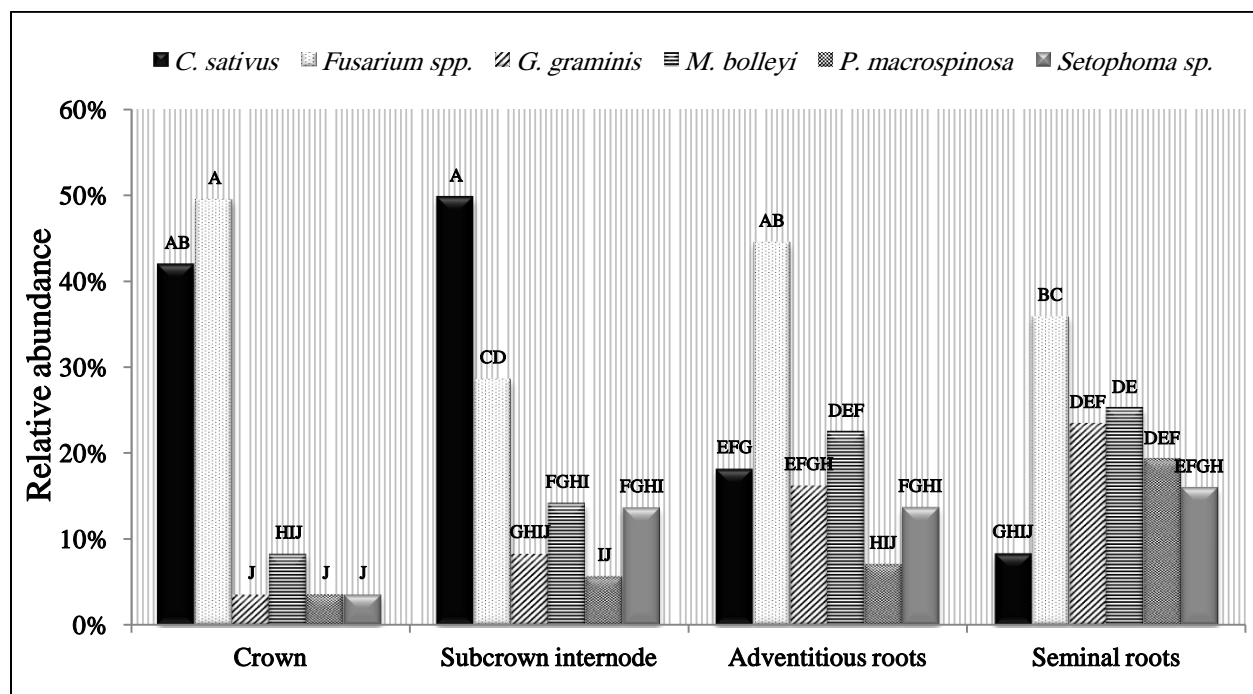


Figure 7.2 Comparative abundance of different fungi isolated from different subterranean organs of wheat. Bars labeled with different letters are significantly different according to LSD at $\alpha = 0.05$, $n = 33$. Data are arcsine square root transformed.

7.5 Discussion

The current study confirmed that different subterranean organs of wheat are different niches occupied by distinct fungal communities. The colonization of subterranean organs by different fungal communities was reported in various plants (Fedel Moen and Harris 1987; Tinline et al. 1994; Vujanovic et al. 2006). Fedel Moen and Harris (1987) in Australia and Tinline et al. (1994) in Canada reported the dominance of *C. sativus* in wheat subcrown internode and crown compared to roots, which agrees with current results. *Cochliobolus sativus*

is a well known leaf pathogen causing spot blotch (Bailey et al. 2003; Duveiller and García Altamirano 2000). It is likely that this fungus is specialized in the infection of leaves and subterranean stems, the subcrown internode and crown (Bailey et al. 2003). In cereals the subcrown internode and the crown are surrounded by basal leaves, which could make the two organs a favorable niche for this species. Chinn and Ledingham (1957) reported the enhancement of *C. sativus* spore germination with the addition of green wheat shoots to the soil, but not with the addition of roots. Duveiller and García Altamirano (2000) have shown that in many cases *C. sativus* isolated from the root and leaves often have similar aggressiveness in causing spot blotch, which supports this hypothesis.

The relatively low abundance of *C. sativus* in adventitious (\simeq 18%) and seminal roots (\simeq 8%) could be attributed to the antagonistic effects of root-inhabiting microbes on this fungus, as suggested earlier (Wildermuth and McNamara 1987), or to the physiological inability of this fungus to compete for resources with soil-borne root pathogens like *Fusarium*, *Gaeumannomyces*, and *Periconia* species.

Most of the comparative studies looking at fungal colonization of the subterranean organs of wheat found uneven spatial distributions of the fungi (Fedel Moen and Harris 1987; Tinline et al. 1994), and in particular of *C. sativus*, but Gonzalez and Trevathan (2000) have also reported *C. sativus* or *M. bolleyi* as most frequent species isolated from all subterranean organs of winter wheat including the root, crown and subcrown internode. However, they sampled wheat at the seedling stage (up to five leaves) and in winter, i.e., under unique environmental conditions, which may explain why their observation contrasts with others.

Many studies looking at the root and crown diseases of wheat did not examine the roots for the quantification of disease or the isolation of causative agents, and it appears that the health status of the subcrown internode and of the crown is usually used as an indicator of root system health (e.g. Fernandez and Jefferson 2004; Fernandez et al. 2011). Many studies on different aspects of disease management, such as yield losses (Ledingham et al. 1973), the effects of crop management on disease (Fernandez et al. 2011; Stevenson and Van Kessel 1996), and even evaluation of genotypes for their resistance to root rot (Tobias et al. 2009) have been done while roots were not included in the analysis. To my knowledge, Tinline et al. (1994) are the only

authors comparing different subterranean organs of wheat for their suitability in common root rot diagnoses. They have used two sets of samples from Saskatchewan and Brazil. In samples from Saskatchewan they observed an inconsistent correlation between the frequency of *C. sativus* in seminal and adventitious roots and disease-severity scores on crowns and subcrown internodes, which raises questions on the usefulness of subcrown internode and crown as indicators of root health. In samples from Brazil, however, disease scores on subcrown internode were highly-correlated with the scores of all the subterranean organs of wheat. Tinline et al. (1994) agree that scoring disease on all subterranean organs gives a more precise picture, but mentioned that subcrown scoring would be “a very useful index of total disease” and is much more convenient than examination of all organs. However, current results have shown that the fungal communities associated with subcrown internode and crown in Saskatchewan are unique and different from that of all other subterranean organs of wheat. The prevalence of *C. sativus* in the subcrown internode and crown, combined with the frequent use of these organs, may have resulted in overestimation of the role of *C. sativus* in root and crown diseases; whereas, the role of pathogens such as *Fusarium* spp. may have been overlooked. My results suggest that despite the suggestion of Tinline et al. (1994), the subcrown internode is not a good indicator of the root mycota and should not be considered representative of root health. I therefore recommend including all subterranean organs in disease studies. Sallans (1965) noticed that the important pathogenic group of *Fusarium* spp. actually received little attention compared with *C. sativus* in studying common root rot disease. Vujanovic et al. (2012) have reported *Fusarium* spp. as the predominant pathogenic fungi in Southwestern Saskatchewan; the results from the present study further emphasizes the importance of this genus in soil-borne diseases of wheat in this region.

Diseases associated with subterranean organs of wheat have been classified according to the identity of the causative agents (Smiley et al. 2005; Wiese et al. 2000). Based on the current study, a likely suggestion might be the definition of diseases based on the affected plant organ. For instance, diseases associated with the subcrown internode and crown could be grouped in one category (e.g. Foot rot) and diseases associated with seminal roots and adventitious roots in another category (e.g. Root rot). Regardless of terminology, I believe that root and crown disease studies in cereals should include all subterranean organs. Precision in describing diseases by

using all organs would lead to a better understanding of the diseases, as suggested by Tinline et al. (1994), and would result in a more successful management of wheat crop health.

7.6 Conclusion

The use of subcrown internode of wheat in the diagnosis of the root diseases has depicted *C. sativus* as the main cause of diseases, concealing the very important involvement of fungi that are specialized to invade root systems, such as species of *Fusarium*. The subcrown internode does not provide a global representation of root health. *Cochliobolus sativus* is by far the most predominant pathogen in the subcrown internode but in seminal and adventitious roots, *Fusarium* spp. is predominant. The examination of all subterranean organs of cereals in the process of disease diagnostics would improve our understanding of root pathology. Furthermore, the definition of the diseases in root and crown disease complex of cereals has been based on the identity of the predominant fungi. The current study suggests that more attention should be paid to the specific preference of pathogens to different subterranean organs.

8. GENERAL DISCUSSION

Durum wheat yield after chickpea is often lower than after pea (Miller et al. 2002). The current study was conducted to understand this phenomenon through changes in root fungal communities. The general hypothesis tested in this research project was that chickpea, or the fungicide application and late termination time which are associated with chickpea production, compromise subsequent durum wheat root health through root associated fungal communities.

8.1 Effect of Fungicides

Fungicide use on chickpea has non-target effects. Most importantly, fungicide use promoted the abundance of *Fusarium* spp. in the roots of a following crop of durum wheat. Increased abundance of *Fusarium* spp. in durum roots may negatively impact durum wheat yield. Many species of *Fusarium* are pathogenic to plants (Bockus and Shroyer 1998; Paulitz et al. 2002; Summerell et al. 2010; Summerell and Leslie 2011). However, it does not seem that the increase in the population of this genus in durum roots could be attributed to a direct effect of fungicides; therefore, other explanations are suggested. Some pathogenic *Fusarium* spp. are stubble-borne (Bockus and Shroyer 1998); therefore, they may not be directly-affected by fungicide residues in soil. Stubble-borne fungi may even benefit from the suppression of their antagonists by fungicide use and/ or from reduced stubble degradation in soil by a saprophytic community due to fungicide use (Robinson et al. 2005; Wildermuth et al. 1997).

Kirkegaard et al. (2004) have shown a decrease in *F. pseudograminearum* infection of wheat crown after brassica rotation was associated with an increase in the population of *Trichoderma* spp. However, pyrosequencing results did not show changes in the population of potential antagonists due to fungicide treatment. Another likely explanation for increased populations of *Fusarium* due to fungicide could be through the indirect effect of fungicide on chickpea. Cruz et al. (2012) have shown a decrease in the production of volatile organic

compounds in chickpea roots with a concurrent suppression effect on pathogenic *Fusaria* due to foliar fungicide application on chickpea.

According to plate culture results, the population of fungal antagonists, including species of *Trichoderma*, *Bionectria*, *Nectria*, and *Myrothecium* (Chatterton et al. 2008; De Schutter et al. 2002; Gülay and Grossmann 1994; Lahlali and Hijri 2010; McQuilken et al. 2001; Rodríguez et al. 2011; Zheng et al. 2011), were reduced in the roots of mature wheat due to fungicide treatment while groups of highly virulent pathogens remained unaffected. This is in agreement with the observation and the explanation that Kirkegaard et al. (2004) have proposed for reduced crown rot disease of wheat after brassica rotation.

The population of antagonists was higher in durum roots after chickpea CDC Luna than CDC Vanguard. Difference in ascochyta blight susceptibility of Desi and Kabuli chickpea cultivars is shown by Gan et al. (2007) which is in agreement with the finding of current study. This suggest that microbial compatibility could be under genetic control, which is supported by other reports (Andreote et al. 2010; Xu et al. 2009). The result suggests that chickpea cultivars differ in their efficiency to enhance root health of the following crop, which may be useful for the industry in choosing chickpea cultivars for rotation.

8.2 Effect of Previous Crop

Fusarium spp. were more abundant in chickpea rotation than pea. Many species of *Fusarium* spp. are plant pathogens (Bockus and Shroyer 1998; Paulitz et al. 2002; Summerell et al. 2010; Summerell and Leslie 2011); therefore, higher abundance of this genus with chickpea rotation may explain lower wheat yield after chickpea. A negative correlation between wheat yield and the abundance of *Fusarium* supports this hypothesis. In Australia, Kirkegaard et al. (2004) showed that using chickpea in rotation increased the abundance of *F. pseudopseudograminearum* in crown compared to brassicas which is in agreement with my current results. However, a correlation does not imply causation and the possibility of other confounding factors exist and need to be researched in future studies. It is very likely that a combination of favorable conditions such as water availability, soil nutrients (Miller et al. 2002) and improved plant health after pea, result in higher productivity of durum wheat after pea.

Kirkegaard et al. (2004) also suggested that reduced cereal stubble degradation in chickpea plots favors *F. pseudograminearum* inoculum abundance. This does not seem to be the case in my study since the amount of previous year surface stubble in pea and chickpea plots was similar in 2009. However, chickpea residues in soil are more durable than those of pea, which means more pulse residues could be transferred to following wheat plots when chickpea is used in rotation (Lafond et al. 2000). This might be a possible explanation for the higher population of stubble-borne Fusaria. As mentioned earlier, the suppression of antagonists when chickpea was used in rotation is another explanation provided by Kirkegaard et al. (2004) for higher incidence of crown rot, but the relative abundance of antagonistic taxa in my pyrosequencing data was very low and there was no difference in their population when pea or chickpea was used in rotations.

Chickpea and pea possess different fungal preferences and defensive compounds (Hadwiger 2008; Stevenson et al. 1997; Ye et al. 2002), which means they may promote different group of microbes. For example, chickpea establishes a rhizobial symbiosis with *Mesorhizobium* while compatible rhizobium of pea is the genus *Rhizobium* (Ahemad and Khan 2010; Prévost and Bromfield 2003). This means the fungal legacy of chickpea and pea to the following crops may be different. Some fungal taxa, such as *Phoma*, *Phaeosphaeria*, *Acremonium*, *Humicola*, and *Cudoniella* were more associated when pea was used in rotation, which is in agreement with this idea. The fact that durum yield after pea is larger than that of chickpea may suggest that these fungi may be beneficial to wheat (e.g. improved mineral nutrition and/or resistance to stressors). Some isolates of *Phoma* have been shown to be beneficial to the host through improved disease resistance and growth promotion (Koike et al. 2001; Shivanna et al. 1996), which supports the above hypothesis. However, it is difficult to judge on the identity and functionality of the aforementioned fungi based on the short ITS sequences provided by pyrosequencing. More research in this regard is warranted.

Pyrosequencing results at fungal phyla level showed an association of Basidiomycota with durum wheat after pea. This group of fungi is known to have a key role in degradation of organic matter especially the complex compounds such as lignin (Frankland et al. 1982; Thormann 2006). It has been suggested that Basidiomycota may play a key role in long term degradation of organic compounds and mineral turnover (Pugh 1980); therefore, their association with pea rotation may be another reason for higher yield after pea. In Saskatchewan, pea is harvested about four weeks

earlier than chickpea (Miller et al. 2001). Over this four weeks, soil in pea plots should be warmer than soil in chickpea plots (Bockus and Shroyer 1998) which could promote growth and reproduction of Basidiomycota.

According to the plate culture data, the population of potentially-beneficial fungal antagonists (Chatterton et al. 2008; De Schutter et al. 2002; Gülay and Grossmann 1994; Lahlali and Hijri 2010; McQuilken et al. 2001; Rodríguez et al. 2011; Zheng et al. 2011) was more abundant in wheat roots after pea than wheat roots after chickpea CDC Vanguard; whereas, highly virulent pathogens were more abundant after CDC Vanguard. There was a negative correlation of yield with the population of highly virulent pathogens. These findings support the initial hypothesis of the study and are generally in agreement with the results of Kirkegaard et al. (2004). These findings may be valuable in planning chickpea rotation in southwestern Saskatchewan.

8.3 Abundance and Comparative Pathogenicity of *Fusarium redolens* in Saskatchewan

Fusarium redolens was frequently isolated from cropping systems of southwestern Saskatchewan, particularly in pulse roots, which may suggest its economic significance. The pathogenicity of this fungus and that of *F. graminearum* and *C. sativus* on pea, chickpea and durum wheat, were tested and it was shown that *F. redolens* was pathogenic on three hosts; however, variation in virulence and susceptibility was observed among fungal isolates and hosts. The abundant presence of *F. redolens* in Saskatchewan shown here, along with the fact that it has been previously reported from Manitoba, Alberta and British Columbia (Gordon 1954; Punja et al. 2007; Sumar et al. 1982) suggests that *F. redolens* is an important component of soil mycota in the Canadian prairies (Esmaeili Taheri et al. 2011). Introduction of pulses to Saskatchewan over the last several decades may also have increased the population of this fungus (Esmaeili Taheri et al. 2011; Knights 2004; Morrall 1997). Further research on biology, ecology and the economic importance of this species in Saskatchewan is warranted.

8.4 Root and Crown Disease Diagnostics in Cereals, Methodological Optimization

Cochliobolus sativus was repeatedly reported as being the prevalent root pathogen in Saskatchewan (e.g. Fernandez and Jefferson 2004; Harding 1973); however, the current study showed the prevalence of *Fusarium* in the root system. To reveal whether the discrepancy is due to using different subterranean organs in disease diagnostic procedures, the fungal communities of different subterranean organs of wheat from different soil types across Saskatchewan were identified. As hypothesized, *C. sativus* was predominant in subcrown internode, the organ often used for root disease diagnostics, and rare in seminal and adventitious roots. Furthermore, the total community of fungi associated with subcrown internode was unique among all subterranean organs of cereals. This suggests that the exclusion of the seminal and adventitious roots from the diagnostic procedure of root rot disease in cereals and focusing exclusively on subcrown internode (e.g. Fernandez and Jefferson 2004; Fernandez et al. 2011; Harding 1973) may underestimate the role of root attacking fungi like *Fusarium*, *Periconia*, *Setophoma*, and so forth. Sallans (1965) noticed that some pathogens have received little attention compared with *C. sativus* in studies of common root rot. Organ specific colonization of fungi has been shown in different studies (Fedel Moen and Harris 1987; Tinline et al. 1994; Vujanovic et al. 2006). Fedel Moen and Harris (1987) reported different fungal species tend toward specific subterranean organs, which is in agreement with the current findings. I recommend including all subterranean organs of wheat in studies of root and crown diseases complex of cereals and applying both culture-based and molecular biology methods, simultaneously.

8.5 Fungicide Growth Inhibition Effects on Relevant Fungal Species

Growth suppression of fungicides on relevant fungal species was tested *in vitro*. The suppression effect of fungicides on different fungal species varied significantly. However, the collective suppression effect on functional group antagonists and highly virulent pathogens was not different; whereas, their abundance was affected differently by foliar fungicide applied in the field. This suggests that indirect mechanisms mediate the effect of fungicides on root-associated fungi in the field. Although more investigation with precise methods is required to draw a firm conclusion, current results suggest foliar application of fungicides may provide an advantage to

some species like *F. redolens* and *F. acuminatum* which are pathogenic (Esmaeili Taheri et al. 2011; Summerell and Leslie 2011).

8.6 The Potentials and Limitations of Pyrosequencing in Field Studies

Despite limitations including short sequence length (Haas et al. 2011), pyrosequencing of the ITS region seems to be a promising method for monitoring fungal communities of environmental samples. It detected higher diversity at the genus level compared to plate culture results. It also covered important phyla of fungi largely-missed by plate culture, namely the Basidiomycota, Glomeromycota, and Chytridiomycota. Furthermore, while plate culture detected no treatment effect in both studies, very interesting community level-effects were detected based on pyrosequencing analyses. This suggests pyrosequencing is a promising method for environmental microbiological studies. While it is still unclear to what extent pyrosequencing data could be considered quantitative (Amend et al. 2010; Sugiyama et al. 2010), current results support its quantitative capabilities as some biologically meaningful results were detected. For instance, the genus *Olpidium* was clearly more abundant in 2010, an unusually wet year, while it was rare during a drier year. Knowing that *Olpidium* possesses swimming spores (Campbell 1996), the need for water to move within the soil shows that pyrosequencing was able to accurately quantify this fungus in the field. Optimization of the high-throughput sequencing methods to yield longer sequences and more precise reference databases (Bridge et al. 2003) will enhance the productivity of metagenomic approaches.

9. GENERAL CONCLUSIONS AND FUTURE RESEARCH

- 1- Fungicide use on chickpea increases the population of *Fusarium* spp. in the seminal roots of a subsequent durum wheat at the heading stage. This could have detrimental effect on plant health and reduce grain yield.
- 2- Fungicide application on chickpea reduced the population of fungal antagonists in symptomatic roots of durum wheat at maturity. Some of the antagonist fungi were shown to effectively suppress the growth of highly virulent fungi in axenic culture; therefore, their suppression by fungicide may negatively affect soil and plant health and translate into yield reduction.
- 3- *In vitro* growth suppression of fungicides varies for different species but the overall suppression effect on functional group antagonists and highly virulent pathogens is not different. This suggests that the effect of fungicide application in the field was the consequence of indirect mechanism(s) rather than direct growth suppression.
- 4- Chickpea CDC Luna enhanced the population of fungal antagonists in the roots of durum wheat more than in those of CDC Vanguard. This shows host genotype is an important factor in shaping fungal community structure and consequently soil and plant health.
- 5- Using chickpea in rotation resulted in a higher population of *Fusarium* spp. in the roots of subsequent durum wheat at heading compared to pea rotation. The abundance of *Fusarium* spp. was negatively related to the yield. This suggests a detrimental effect of *Fusarium* spp. on plant health and may explain the poor performance of wheat after chickpea.
- 6- Several fungal taxa, such as *Phoma*, *Phaeosphaeria*, *Acremonium*, *Humicola*, and *Cudoniella*, were more abundant in durum root after pea. Higher durum yield after pea may suggest beneficial effects of these fungi on growth. Possible mechanisms may include improved mineral nutrition, biological or environmental stress tolerance, and phytohormone production.

- 7- Using pea in rotation is associated with higher populations of Basidiomycota in the roots of subsequent durum wheat. Basidiomycota are known for their key role in degradation of complex compounds like lignin (Robinson et al. 2005; Thormann 2006), which subsequently may be important in nutrient turnover in the soil (Pugh 1980). This may further explain better performance of wheat after pea.
- 8- Using pea in rotation increased the population of fungal antagonists in the mature roots of a following durum wheat crop compared to chickpea. Under controlled conditions, these fungi showed a mycoparasitic relationship to highly virulent root pathogens, which may further explain why pea is a more efficient rotation crop.
- 9- Chickpea CDC Vanguard resulted in a higher population of highly virulent pathogens in the roots of a subsequent durum wheat. This suggests the possibility of using selected crop genotypes to manage plant health in agro-ecosystems.
- 10- Early chickpea termination (at pea maturity) did not affect the community of culturable fungi associated with subsequent durum wheat either at the total community level or at the functional group level.
- 11- The diagnoses of the root diseases of cereals has usually been restricted to the examination of the subcrown internode (e.g. Fernandez and Jefferson 2004; Fernandez et al. 2011; Harding 1973). Current results show that the fungal communities associated with subcrown internode are unique and not representative of the whole root system. Consequently, the current approach does not cover the wide spectrum of fungi involved in the disease complex and the role and economic damages caused by some key pathogens (e.g. *Fusarium* spp.) may have been overlooked, while *C. sativus* has received too much attention.
- 12- *Fusarium redolens* has not previously been reported in Saskatchewan. Current studies suggest that this fungus is an important component of the soil mycota associated with pulse roots in Saskatchewan.
- 13- Species of *Fusidium*, *Cladosporium*, *Lachnum*, *Setophoma* and *Ophiosphaerella* were more abundant in non-symptomatic roots, and may benefit the plant by preventing diseases or enhancing tolerance to abiotic stresses. This hypothesis warrants future research.

- 14- Organization of fungal species with similar functionality in the ecosystem seems to be promising in detecting important environmental effects in microbial community studies. It improves statistical power and predictive ability of the results (Olof et al. 1990; Zak and Visser 1996).
- 15- Despite limitations including short sequence length (Haas et al. 2011), 454 pyrosequencing seems to be a promising approach in metagenomic studies of microbial communities in field level.

This research showed that using chickpea in rotation and fungicide application on preceding chickpea may negatively impact root health parameters in following durum wheat crop through changes in fungal community. For instance, an increase in the population of *Fusarium* spp. and a decrease in the population of the fungal antagonists including four species of *Trichoderma*, one species of *Bionectria*, one species of *Nectria* and one species of *Myrothecium* (Chatterton et al. 2008; De Schutter et al. 2002; Gülay and Grossmann 1994; Lahlali and Hijri 2010; McQuilken et al. 2001; Rodríguez et al. 2011; Zheng et al. 2011) were observed in wheat roots due to fungicide use on a previous crop of chickpea. According to the results of two field studies, there was a positive correlation between functional group of antagonists and durum wheat yield ($r_s = 0.297$, $P = 0.011$) and a negative correlation between antagonists and highly virulent pathogens ($r_s = -0.281$, $P = 0.016$), which suggests the relevant involvement of antagonists in biocontrol of root diseases in field condition. Most of the antagonist fungi were shown to be mycoparasites of well known root pathogens *in vitro* (data not shown). Many species of antagonistic fungi are commercially used as biocontrol agents (Harman 2000; Liu et al. 2010; McLean et al. 2012; Moradi et al. 2012) therefore, an attractive approach would be to study the effect of isolated indigenous antagonists in the greenhouse and under field conditions in the search for potential biocontrol and biofertilizers.

Healthy roots of durum wheat were usually colonized by fungal endophytes (mostly dark septate). Some endophytic fungi are known to improve plant nutrition, disease and environmental stress tolerance (e.g. Jumpponen et al. 1998; Khan et al. 2011; Lahlali and Hijri 2010; Li et al. 2012; Newsham 1999; Ownley et al. 2008; Shukla et al. 2012); therefore, more studies on greenhouse and field inoculation of these fungi are warranted. The same applies to the

fungi present at the heading stage of durum following pea (e.g. *Phoma*, *Phaeosphaeria*, *Acremonium*, *Humicola*, and *Cudoniella*), as we know that the yield of a following crop of durum usually is better after pea.

The *in vitro* study of fungicide growth suppression showed that the effect of fungicide observed in the field may not be completely explained through a direct growth-suppression effect of the fungicides. Consequently, indirect mechanism(s) may be involved. I think in regard to functional group response to fungicides, trophic niche specialization of each functional group is the key point. Accordingly, I suggest the main reason for no effect of fungicides applied in the field on highly virulent root pathogens is the fact that they may be specialized to inhabit roots; therefore, their dependency on soil organic carbon sources and soil conditions is minimized (Garrett 1951). This means while the majority of soil inhabiting fungi including fungal antagonists are completely dependent on living conditions in the soil and are highly vulnerable to fungicide disturbance (Robinson et al. 2005), the highly virulent pathogens may take refuge in pulse and/ or cereal stubble where they are “protected” from fungicide pollution. Suppression of soil inhibiting saprobes, on the other hand, slows down degradation of stubble which in turn saves the exclusive niche of highly virulent pathogens. The above mentioned hypothesis is supported by the fundamental and very interesting concept of “ecological groups of soil fungi” introduced to soil fungal ecology decades ago (Burgess 1939; Garrett 1951). According to this theory, fungi in the soil could be divided into a few major “ecological groups” according to their ability to consume carbon sources (Burgess 1939; Garrett 1951). Intraspecific variation in trophic specialization of some fungi is observed, and trophic habit of a specific isolate may change in relation to carbon source (Gordon and Martyn 1997; Termorshuizen and Jeger 2008); therefore, I suggest precise trophic studies on highly virulent pathogens and their fungal antagonists in Saskatchewan agro-ecosystems. This will help us understand the life cycle of these important fungi in the Canadian prairie and enable us to manage them in a more sustainable manner.

Basidiomycota and Chytridiomycota remained largely undetected by plate culture while pyrosequencing proved their abundance in durum roots. It is suggested that culture based methods with readily available sugar sources are usually not favorable for these fungi (Garrett 1951); therefore, it is not surprising that their functionality and importance remained

ignored in most fungal ecology studies (Pugh 1980). Basidiomycota are important in degradation of complex compounds like lignin (Frankland et al. 1982) and I completely agree with Pugh (1980) that their role in nutrition to plants may be very critical and considerably-overlooked or even ignored, especially in agro-ecosystems. Revealing biodiversity and functionality of Basidiomycota in agricultural soil will be essential for implementation of sustainable agriculture. Chytridiomycota on the other hand are reported to be involved in root disease (Hartwright et al. 2010), and especially the transfer of viruses among hosts (Campbell 1996). Their economic damages to the yield may be significant, especially in wet years.

This research looked at the fungal communities associated with durum wheat roots as influenced by the crop and the cropping practices used the previous year. It would be very interesting to study the community of fungi associated with the previous pulse crop. This would help us understand how the effects of treatment seen in wheat have developed through time.

Among the three fungicides used in this study, Lance was the most persistent in soil (Yang et al. 2012b). This may suggest a prolonged effect of this fungicide in the environment. I suggest closer investigation on degradability and effect of Lance in agro-ecosystems and even considering replacing this fungicide with faster-degrading compounds.

Fusarium was a major component of wheat mycota at the heading stage (about 30% of mycota). The ecological and economical significance of this fungus in Saskatchewan may have been overlooked mainly due to the methodological bias of using subcrown internode in root rot severity quantification and the isolation of pathogen (e.g. Fernandez and Jefferson 2004; Fernandez et al. 2011). This research shows that *Fusarium* may be favored by using chickpea in rotation and fungicide application. Many species of *Fusarium* are mycotoxin-producers which could be a threat to food safety (Sweeney and Dobson 1998). Translation elongation factor-1 alpha is usually believed to provide a better resolution for the identification of *Fusarium* species (O'Donnell et al. 1998); therefore, precise monitoring of Fusaria in this agro-ecosystem using more specific primers, like the Elongation factor, is warranted.

Soil and root health status are very important in agro-ecosystems. In fact, one of the primary goals of crop rotation in cereal-based agro-ecosystems is to minimize the effect of soil-borne disease (Baker and Cook 1974; Cook 2007; Janvier et al. 2007). However, it is surprising that soil-borne diseases did not receive much attention in rotation studies in this region. Studies that tried to investigate plant health status in relation to farming management usually relied on the classic subcrown internode method of disease quantification and pathogen isolation, overlooking the health status and fungal community associated with seminal and adventitious roots (e.g. Fernandez et al. 2011; Stevenson and Van Kessel 1996). My results showed that the conventional method of diagnosis does not provide a comprehensive picture of pathogens associated with subterranean organs and plant health status. In fact, at the beginning of this study, a subcrown internode-based quantification of root rot severity was done on samples of 2009 and no effect of rotation crop and fungicide was detected. Consequently, if the community of fungi associated with seminal and adventitious roots would not have been examined using pyrosequencing, no effect would have been detected and an erroneous conclusion would have been made. I strongly recommend including all subterranean organs of cereals in studies requiring root disease quantification. Also, based on the strong bias of culture-based methods against important fungal phyla such as Basidiomycota and Chytridiomycota with essentially no coverage of Glomeromycota, I recommend using at least one molecular-based technique in studies of fungal communities in the field.

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